GENETIC DISSECTION OF PHYTOCHROME A SIGNAL

TRANSDUCTION IN ARABIDOPSIS

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

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March 2000
Acknowledgements

I would like to thank my supervisors Prof. Harry Smith and Prof. Garry C. Whitelam for their support of this Ph.D. Dr. Uta Praekelt who, in addition to isolating the mutant *gill*, has patiently provided endless support and advice in the laboratory. Dr. E. Rosato introduced me to cloning and was extremely helpful with associated troubleshooting. I would also like to thank the departmental technical staff for their commitment to weekend watering, mending cabinets, autoclaving etc. Many members of the Biology department (past and present) have been good colleagues and friends over the duration of this Ph.D.

I would like to thank Paul Devlin for his work on *fhy3* (detailed in text) and Dr. P. Puente for providing new alleles of *fhy3*. Finally I would like to thank my family and Tony, for their moral and financial support through this Ph.D., particularly the last few months.
Abstract

The signalling pathway of phytochrome A (phyA) is complex. This thesis describes analysis of mutants with altered phyA-mediated responses for enhancement of understanding concerning phyA signalling.

A novel Arabidopsis mutant (gill) has been isolated which defines a point of interaction between phytochrome signalling and gravity signalling. This mutant, in contrast to wild-type (wt), displays gravitropic orientation of hypocotyls under red (R) and far-red (FR) light. The phytochrome mediated agravitropism, observed in wt under R and FR, is mediated by phyA and phytochrome B (phyB) (Poppe et al, 1996, Robson et al, 1996). Analysis of the gill mutant phenotypes suggests that this mutant is impaired in both phyA and phyB mediation of agravitropism.

The T-DNA insertion, which is most likely responsible for the phenotype associated with gill, was located to chromosome V, between two genes predicted by sequence analysis. Reduced expression of one of these genes (K19M22.14) occurs in gill. Database analysis of the sequence of the K19M22.14 gene suggests no homology to any recognised genes.

The phyA signalling mutant fhy3 (Whitelam et al, 1993) has diminished responses to FR but amplified responses to R. The phenotypes associated with these amplified responses to R are here characterised for a number of different alleles in three ecotypes of Arabidopsis. Results suggest that fhy3 has some enhanced responses to R, and that these are more apparent in some alleles/ecotypes. Analysis of the fhy3phyB double mutant indicates that phyB is required for the enhancement of R responses through FHY3.

Isolation and preliminary characterisation of mutants (sofs) which suppress some phenotypes of fhy1 is presented. These mutants may define new components of the phyA signalling pathway.
Abbreviations

B - Blue light
CAB - Chlorophyll a/b binding protein
CaMV - Cauliflower mosaic virus
CHI - Chalcone isomerase
CHS - Chalcone synthase
d - Day
EMS - Ethyl methane sulfonate
FR - Far-red light
GFP - Green fluorescent protein
GWP - Genome walker product
h - hour
HIR - High irradiance response
kDa - Kilodalton
LD - Long day
LFR - Low fluence response
M - Molar
min - Minute
mol - Mole
NR - Nitrate reductase
ORF - Open reading frame
pBS - Bluescript vector
PC - Plastocyanin
PCR - Polymerase chain reaction
phyx - Biologically active phytochrome x
PHYx - Phytochrome x gene
POR - Protochlorophyllide oxidoreductase
R - Red light
RBCS - Small subunit of ribulose-1,5-bisphosphate carboxylase
SD - Short day
VLFR - Very low fluence response
Abbreviations

W - White light
WT - Wild type
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Introduction

1.0 Introduction

The acquisition of information concerning the surrounding environment is fundamental for plant survival. With no option to escape, plants must develop in such a way as to utilise the resources available in an ever-changing environment, in the most effective manner. Light is one of the major factors that influence these developmental adaptations. Plants have evolved a series of photoreceptors, which enable the plant to detect light and elicit different responses according to signals of quality, quantity, period and direction. The photoreceptors can be divided into four groups; the phytochromes, detectors of red (R) and far-red (FR) light; the cryptochromes, detectors of blue (B) and UV-A; the UV-B detectors; and the phototropins which detect B, UV-A and green light. The change in the pattern of development in response to light is termed photomorphogenesis and is independent from the process of photosynthesis.

1.1 Photoreceptors

1.1.1 UVA, UVB and blue light photoreceptors

Both UV-A and UV-B inhibit axis elongation, demonstrating that plants contain photoreceptors for these wavelengths of light. A number of responses are controlled by blue light; for example inhibition of axis elongation, phototropism, stomatal opening, cell division and chloroplast movement in ferns, and carotenoid synthesis in Neurospora (Kendrick and Kronenberg, 1994). The blue light photoreceptors cryptochrome 1 (CRY1) and cryptochrome 2 (CRY2) have homology to photolyases, but do not display the photoreactivation activity that is typical of photolyases, *in vitro*. The cryptochromes lack a specific residue that is important for photolyase DNA substrate recognition (Ahmad *et al*, 1997). There is also an uncharacterised UVB photoreceptor in Arabidopsis (Schäfer *et al*, 1997). UVB (280-320nm) may cause damage to DNA, but at reasonable levels it can have morphogenic effects, and induces expression of genes for UV protective pigments (Jenkins, 1997). Phytochromes can also absorb these wavelengths of light so analysis of specific photoreceptors is complicated.
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1.1.2 Phytochromes

The phytochrome family of photoreceptors detects light in the red (R) to far-red (FR) range (650-800nm). Phytochromes exist in two interconvertible forms. Phytochrome is synthesised in the physiologically inactive form (Pr) which absorbs maximally in the R region of the spectrum. Conversion to the active Pfr form occurs following the absorption of a photon. The Pfr form is photoconverted back to Pr by the absorption of a photon, with maximal absorption occurring in FR at around 730nm. Continuous broad band irradiation establishes an equilibrium between Pr and Pfr.

1.2 Phytochrome structure and regulation

In Arabidopsis there are five phytochrome genes PHYA-PHYE (Clack et al, 1994). These have been mapped to four of the five Arabidopsis chromosomes with PHYD and PHYE both located on chromosome 4 (Matthews and Sharrock, 1997). The polypeptide products of the PHY genes have the same overall structure, despite differing significantly in sequence. Their relatedness suggests they may have similar signal transduction mechanisms. Phytochrome D shows a high similarity with phytochrome B and divergence of PHYB from PHYD follows a recent gene duplication. Low transcript levels of phytochromes D and E suggest they are likely to be the least abundant (Clack et al, 1994). All dicotyledonous plants investigated to date have five phytochrome genes, but monocotyledonous plants may lack PHYE. This suggests that PHYE diverged from the other phytochromes after the divergence of monocots from dicots. PHYE is most likely to have diverged from the PHYB/PHYD branch.

Phytochrome molecules are homodimers, with each polypeptide monomer being approximately 125 kDa in size. The phytochromes have the same basic primary structure as each other. The polypeptide chain consists of two domains; the N-terminal domain, which has a site for covalent thioether linkage of a linear tetrapyrrole chromophore (phytochromobilin (PΦB) synthesised from haem in the plastid) and which cradles the chromophore in a hydrophobic pocket; and the C-
terminal domain which is more extended (Quail, 1997). The C-terminal domain is responsible for the non-covalent dimerisation of the molecule, and for transmission of perceived photosignals (Quail et al, 1995).

The photoconversion of phyA to Pfr leads to its rapid degradation. Turnover of PfrA is 100-fold greater than PrA, and since Pfr is only present after photoconversion of Pr, phyA is known as a “light-labile”. Light-labile phytochromes have alternatively been named as Type I phytochromes. Pfr levels of phytochromes B-E are relatively unaffected by transfer to light and so these are “light-stable” or Type II phytochromes.

The loss of phyA Pfr occurs with loss of immunochemically - and spectrophotometrically-detectable phyA, so it is not merely due to loss of the chromophore or denaturation of the chromoprotein. Not only is the Pfr form degraded but a proportion of molecules that have been cycled through Pr→Pfr→Pr can also be destroyed. The degradation of Pfr is an enzymatic process and is therefore temperature dependent (Schäfer and Schmidt, 1974). A certain amount of Pfr is protected from degradation by a non-photochemical reaction- dark reversion, in which Pfr is converted to Pr. Dark reversion is evident in etiolated dicot seedlings (Centrospermae e.g. *Amaranthus* are exceptions, (Kendrick and Frankland, 1968)) but absent in cereals (Vierstra, 1994). The degree of dark reversion depends on the initial photostationary state, ([Pfr]/[Ptot]), (Schmidt and Schäfer, 1974). Dark reversion and decay do not appear to compete for the same pool of Pfr (Schäfer and Schmidt, 1974).

The ability to regulate the levels of phytochrome within the plant is an important requirement, demonstrated by the changes in morphology that occur in plants with reduced or overexpressed levels. This regulation is achieved by controlling the synthesis and stability of the phytochromes. The regulation of phyA is a good example; its level is determined by control of *PHYA* gene transcription, mRNA
stability, protein availability and protein turnover. The largest effective control of phyA levels is the rapid proteolysis of Pfr.

1.2.1 Heterodimers/homodimers

The fluence required by phyA and phyB for a similar response is different by about four orders of magnitude (Furuya and Schäfer, 1996). A number of possible reasons for this can be proposed, such as different binding affinities, the difference in relative population sizes or different response amplification factors. Furuya and Schäfer (1996) propose that the activity of phytochrome may be mediated differentially by heterodimers and homodimers. Phytochrome A is proposed to act in the heterodimer form PrAPfrA, whereas phyB is only active in the homodimer form PfrBPfrB. The PrBPrB homodimer form is thought to be inactive. The heterodimer form of phyB would be unable to activate or inactivate signalling in this model. It is thought to be the heterodimer pool of phyA, (PrAPfrA) that displays dark reversion. Heterodimers are formed in etiolated seedlings under very low fluence R, whereas the homodimer pool is wavelength dependent. The difference in sensitivity of phyA and phyB may arise if phyB is only active in the homodimer form; this would also account for the R/FR reversibility of phyB responses (Furuya and Schäfer, 1996).

1.2.2 Nucleocytoplasmic partitioning of phytochrome

The distribution of phytochrome within the cell may be critical for its function. Immunochemical analysis of isolated Arabidopsis nuclei and GUS-phyB staining located endogenous phyB in the nucleus (Sakamoto and Nagatani, 1996). Nuclear phyB levels were reduced in dark adapted plants irradiated with FR, suggesting the nuclear localisation of phyB to be light-dependent. Recent experiments by Kircher et al (1999) have demonstrated, using phytochrome-GFP fusions transgenically expressed in tobacco, light-dependent translocation of phyA and phyB from the cytosol to the nucleus. In dark-grown and dark-adapted plants phyB-GFP is located in the cytosol; continuous R or R pulses lead to nuclear localisation of the phyB-GFP fusion in a R/FR reversible manner, so the localisation is a function of the
phytochrome photoconversion. Nuclear import of phyA-GFP is also light-dependent, occurring in the presence of a R light pulse or a FR pulse.

Import of phyA and phyB may occur via different mechanisms as the kinetics of import are different. Once in the nucleus phyA and phyB show different distribution patterns. Neither is evenly distributed, but they each form a different number and size of “spots” (Kircher et al, 1999). The pattern of distribution indicates that within the nucleus phyA and phyB probably interact with different proteins.

The translocation of phytochromes into the nucleus leads to new proposals for the action of phytochrome. Interaction with nuclear proteins, for example, PIF3 (phytochrome interacting factor 3), to form multiprotein complexes may occur which in turn could regulate gene expression directly. This direct regulation by photoreceptors has already been observed with cryptochrome in mice (Shearman et al, 2000) and Drosophila (Ceriani et al, 1999).

1.3 Phytochrome response modes

Phytochrome has a number of response modes to light, which have been classified according to fluence and the period of irradiation:

1.3.1 The Low Fluence Response (LFR): this response is characterised by the classic R/FR reversibility of phytochrome first observed by Borthwick et al (1954). The LFR obeys the Bunsen-Roscoe reciprocity law; i.e. the response is proportional to the quantity of photoproduct, this can be produced by either low photon irradiance for long periods, or by high photon fluence for short periods. This response is saturated by brief pulses and at low fluences, the response being activated from 1 \( \mu \)mol m\(^{-2}\) red light, which in etiolated seedlings is calculated to convert \( \sim 2\% \) of phytochrome. Increasing dark intervals between R and FR lead to an escape from reversibility, as enough time elapses in this dark period for Pfr action.
1.3.2 The Very Low Fluence Response (VLFR): This extremely sensitive response requiring 0.1 nmol m\(^{-2}\) or less R can only be observed in dark-grown, imbibed seeds or seedlings in which only Pr is present. This response shows no R/FR reversibility as it can be mediated by FR, requiring very few molecules of Pfr for saturation. The Bunsen-Roscoe law of reciprocity is still obeyed.

1.3.3 The High Irradiance Response (HIR): The HIR is dependent on fluence rate and prolonged periods of exposure, and therefore does not show reciprocity. The HIR occurs in R, FR, B, UVA and UVB. The extent of the HIR is a function of the fluence rate, exposure time and wavelength. Although prolonged exposures are required for the function of the HIR, continuous exposure is not necessary; cyclic irradiations of equal fluence rate, over the same time period as the continuous irradiation, are sufficient to mediate the same response if the periods of dark are of limited time. Examples of HIR include anthocyanin synthesis and seedling survival in shade conditions where the FR-HIR of phyA acts to prevent extensive elongation.

Interaction between the HIR and LFR occurs. The effect of the R/FR reversible LFR in etiolated seedlings can be enhanced by prolonged light treatments with respect to inhibition of hypocotyl elongation, cotyledon expansion and anthocyanin production (Mancinelli, 1994). De-etiolation responses such as inhibition of hypocotyl elongation, hook opening and cotyledon expansion are mediated by each of the different response modes.

1.4 Phytochrome signalling pathways
The developmental response of an organism to a stimulus is generated by the transmission of information from a receptor which perceives the stimulus, sometimes via effector proteins and/or second messengers to elicit the response. These pathways, and their regulation in plants, are different from those in animals which have less developmental flexibility, the ability to adapt to the environment being less critical in animals which are able to move away from unfavourable conditions. A number of techniques have been utilised to elucidate the molecules
which carry out the signal transduction from phytochrome; these include microinjection of intermediates into phytochrome deficient cells, using chemicals which act as agonists or antagonists to these pathways, and using antibodies to cell signal components known to exist in animals.

1.4.1 Signal pathway components

The tomato *aurea* mutant is deficient in phytochrome chromophore synthesis. Injection of *aurea* mutant cells with intact phyA restored anthocyanin biosynthesis and chloroplast development in these cells; the signalling for these responses was cell-autonomous and so the neighbouring cells showed no restoration of the wild type phenotype (Neuhaus *et al.*, 1993). These experiments demonstrated that phyA mediated these events and provided a system for analysis of responses from putative signalling intermediates. Heterotrimeric G-proteins are early components of the signal transduction pathway, determined using agonists and antagonists of G-protein pathways (Neuhaus *et al.*, 1993, Bowler and Chua, 1994), with Ca$^{2+}$ and cGMP (cyclic GMP) being responsible for downstream events via different pathways. The roles of Ca$^{2+}$ and cGMP in the phyA signal transduction pathways have been determined to some extent (Bowler *et al.*, 1994a, Bowler *et al.*, 1994b, Neuhaus *et al.*, 1993). Cyclic GMP mediates phyA-dependent anthocyanin biosynthesis by controlling expression of, for example, the gene encoding chalcone synthase (CHS), an enzyme in the anthocyanin biosynthesis pathway. Partial chloroplast development is also induced by cGMP. Synthesis of a full complement of photosynthetic complexes for complete chloroplast development requires a combined induction by the cGMP and Ca$^{2+}$ pathways. Ca$^{2+}$ via calcium-induced calmodulin controls chloroplast development via, for example, the synthesis of chlorophyll a/b binding protein (CAB) of photosystem II. It could be suggested from the experiments described above that Ca$^{2+}$ and cGMP appear to be the only signalling molecules involved in phyA-dependent anthocyanin biosynthesis and chloroplast development (Bowler *et al.*, 1994b). There are, however, limitations to these experiments; phyA may, to some extent, exert divergent roles in different species; additionally, the role
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of endogenous phyA could be more complex than that observed for injected phyA, which may not be subject to the same regulation by other cellular components.

The activity of many signal transduction components, at least in animal cells, is regulated by phosphorylation and dephosphorylation of serine (Ser), threonine (Thr), or tyrosine (Tyr) residues. This is mediated by protein kinase cascades such as the mitogen activated protein (MAP) kinase cascade. The components for a MAP kinase cascade are present in plant cells. It has been speculated that phosphorylation of a Ser residue in the N-terminal domain of phyA may cause a local conformational change and so trigger signal transduction. Evidence for this arises from the phosphorylation of a conserved N-terminal Ser residue of oat phyA in vitro (Wong et al., 1986). Phosphorylation/dephosphorylation of some proteins can be instigated via phytochrome by R/FR (Mösinger and Schäfer, 1984). Etiolated oat seedling extracts and nuclear preparations have demonstrated phytochrome-modulated protein kinase activity (Song et al., 1997). From recent work, Yeh and Lagarias (1999) conclude that eukaryotic phytochromes are serine/threonine protein kinases for a number of reasons. Recombinant phytochrome expression in yeast systems displays protein kinase activity; the biochemical properties of this activity are similar to those of phytochrome isolated from plants. Phytochrome concentration does not affect ATP-dependent phytochrome phosphorylation; autophosphorylation of phytochrome is modulated by chromophore and light.

The process for the transfer of information from photoreceptors to initiate a signal transduction reaction has until recently been limited to that of cGMP and Ca²⁺ pathways. However, Ni et al. (1998, 1999) have identified a phytochrome-interacting factor (PIF3). PIF3 interacts with the C-terminal domains of both phyA and phyB and so acts immediately downstream of both photoreceptors. Phytochrome B only binds PIF3 when in the Pfr form and the interaction is R/FR reversible. PIF3 is a putative basic helix-loop-helix (bHLH) protein, a class of transcriptional regulators; this structure would enable phytochrome to directly affect photoregulated genes. In
support of this PIF3 also contains a bipartite nuclear localisation signal (NLS) and is localised in the nucleus.

Another protein, PKS1 (phytochrome kinase substrate 1), which binds to the C-terminal domains of phyA and phyB, has also been identified (Fankhauser et al, 1999). PKS1 binds to both the Pr and Pfr forms of phytochrome. The location of PKS1 in the cytoplasm has been confirmed by GFP fusion analysis. PKS1 appears to be phosphorylated by phyA in the same light-dependent manner as the phyA autophosphorylation. A role for PKS1 in negative regulation of phyB signalling has also been proposed.

NDPK2 (nucleoside diphosphate kinase 2), may also be a component of the phytochrome signalling pathways of Arabidopsis and a role for NDPK2 has been demonstrated in both the phyA mediated FR-HIR and the phyB mediated R-HIR (Choi et al, 1999). NDPK2 preferentially binds Pfr in vitro, and in the same way as for PIF3 this may provide a R/FR reversible on/off switch for signalling.

It is difficult to reconcile the existence of cGMP and Ca\(^{2+}\)/calmodulin pathways with the presence of components, such as PIF3, which interact with phytochrome for direct regulation of genes. Phytochrome could interact with PIF3 and maybe other components, such as NDPK2, to form a multiprotein complex for gene regulation. The components may vary according to the phytochrome (A-E) binding, therefore causing regulation of different genes. Alternatively, the components may be constant, with specificity of interaction determined only by phytochrome. This direct gene regulation may be ideal for responses to light which occur quickly and for induction of short-lived proteins for which continual gene induction is required. The role of the cGMP and Ca\(^{2+}\)/calmodulin pathway may be for slower reactions which result in more long-term changes within the plant, probably exerting a greater effect on plant morphology. The interaction of Pfr/PIF3 could regulate a cascade of genes, one of which may induce changes in levels of components of the Ca\(^{2+}\)/calmodulin pathway or cGMP pathway, and in this way phytochrome could control responses of
these pathways. Phytochrome may play different roles in the cytoplasm and the nucleus; so direct interaction of phytochrome with Ca$^{2+}$ or cGMP may occur in the cytoplasm for control of these pathways, while phytochrome transferred to the nucleus may regulate gene expression.

1.4.2 Reciprocal control

The term reciprocal control was proposed by Bowler et al (1994b) to describe the phenomenon of a positive regulator of one signal transduction pathway being able to exert negative control over another. This regulation has been observed in the calcium/cGMP pathways. Inhibition of the calcium pathway causes hyperstimulation of CHS expression, and artificial stimulation of CHS is accompanied by decreased CAB mRNA levels (Bowler et al, 1994b). This is the first negative feedback loop observed in phytochrome signal transduction, other than down-regulation of signalling by Pfr destruction (Schäfer et al, 1997).

1.4.3 Chalcone synthase gene expression

At least in Arabidopsis, mustard and parsley the photoreceptor that mediates CHS mRNA induction is dependent on the developmental stage of the plant. The expression of CHS in mature leaves is regulated mostly by blue/UV, mediated by CRY1, whereas in the seedling or in dark grown plants phytochrome is the principal regulator (Frohnmeyer et al, 1992). The control of CHS expression occurs via different photoreceptors in different species. In Arabidopsis phyA is responsible for CHS mRNA accumulation, but in the absence of phyA and phyB the blue, UVA and UVB photoreceptors can regulate expression (Batschauer et al, 1996). In mustard both phyA and phyB may play a role in CHS expression, as accumulation of CHS mRNA occurs following R or FR light. In parsley cell cultures R cannot induce CHS expression, but receptors of blue UVA and UVB appear to be involved. The signal transduction chains from different photoreceptors merge to activate transcription factors at a 52 bp light regulated unit on the CHS promoter (Kaiser et al, 1995). There is evidence that the transcription factors may be located in the cytosol, with
their transport into the nucleus allowing them to activate their targeted promoter (Schafer et al, 1997).

Using parsley cell suspension cultures, translocation of a bZIP protein from the cytosol to nucleus has been observed to be dependent on light quality, suggesting this may be an important step in the signal transduction cascade (Schafer et al, 1997). cGMP is responsible for phytochrome regulation of CHS expression, however CHS expression induced by UVB occurs via the Ca\(^{2+}\)/calmodulin pathway. Temporal separation of the R cGMP pathway and the UV Ca\(^{2+}\)/calmodulin pathway for CHS regulation (the cGMP pathway functioning first) has been demonstrated in soybean (SB-P) photomixotrophic cell suspension cultures (Frohnmeyer et al, 1998). Protein phosphatase and serine/threonine protein kinase activity are required for blue/UVA and UVB induction of CHS (Christie and Jenkins, 1996). Expression of CHS by UVB is enhanced synergistically by the signal transduction pathways of blue and UVA.

1.4.4 CAB expression

The expression of the gene family for the chlorophyll a/b binding protein (CAB) is often used as a measure of response of a plant to particular light conditions. A reporter gene such as luciferase (LUC) can be used to measure this response. Expression of CAB occurs naturally in a circadian manner, peaks of expression in tobacco occurring at 4, 18 and 48 hours after a pulse of R (Millar et al, 1992). In Arabidopsis the induction of CAB expression is mediated by both phyA (in response to very low fluence light) and phyB (in response to low fluence light); phyA is the more sensitive photoreceptor for CAB induction. The phyA-specific induction is photoreversible whereas phyB-specific induction is photoreversible. phyA and phyB are not the only photoreceptors involved in CAB induction; at least one more phytochrome controls expression in a photoreversible manner. It is proposed that phyA induces CAB expression in etiolated seedlings, masking the effects of phyB and the unidentified phytochrome (Hamazato et al, 1997). However,
phyB does play a role in etiolated seedlings to regulate positively the level of expression of circadian oscillations (Anderson et al, 1997).

1.5 Aspects of plant development mediated by phytochrome

1.5.1 Shade-avoidance

The green tissues of plants absorb much more R than FR as well as absorbing B, so light transmitted or reflected from these tissues has a very different spectrum to that of natural daylight. The ratio of R to FR light can be determined by a plant, enabling it to detect any competitors which are shading it. In response to shade many plants, although not all, have an altered pattern of growth. Examples of shade avoidance phenotypes include seedling elongation, increased petiole and leaf expansion, greater apical dominance, altered leaf angle, reduced chlorophyll synthesis and accelerated flowering. The shade-avoidance syndrome is predominantly mediated by phyB; however, loss of phyA, phyD and phyE also lead to changes in these responses. Plants can detect the presence of neighbours and respond before they are shaded by them. The early warning of nearby plants comes from the FR light reflected by their leaves. Experimental evidence using plants exposed to altered R:FR ratios but with the same available PAR (photosynthetically active radiation), supports this theory (Ballaré et al, 1997, Smith, 1982, Smith, 1994). Shade avoidance can only occur when enough resources are available for accelerated growth; for this sufficient PAR needs to be received. Similarly, shade-avoidance must be prevented in young seedlings until sufficient reserves are accumulated; the phyA FR-HIR may provide such a mechanism until phyA is lost (Smith et al, 1997, Yanovsky et al, 1995). Germination is also inhibited by a low R:FR ratio in some plants, a secondary dormancy being imposed that prevents germination under shade conditions (Smith and Whitelam, 1997).

1.5.2 Photoperiod and phytochromes

In Arabidopsis, phyA plays a significant role in daylength perception. In long day plants (LDP) flowering is promoted when a R day extension is interrupted with FR;
the degree of response to this FR follows a circadian rhythm, suggesting interaction of phyA and the photoperiodic clock occurs (Jackson and Thomas, 1997). A role for phyA in short day plants (SDP) has not been demonstrated; however, a role for phyB in the determination of sensitivity to photoperiod has been shown in *Sorghum* flowering (Pao and Morgan, 1986). Photoperiod perception by phyB has also been demonstrated in potato which will only tuberize in SD (Jackson *et al.*, 1996). Reduced expression of phyB in potato leads to day-neutral tuberization. Photoperiod perception for the induction of flowering in Arabidopsis has been shown to be regulated by CRY2 (Guo *et al.*, 1998).

Gibberellins have been implicated in the control of photoperiodic responses (Jackson and Thomas, 1997) and other factors such as brassinosteroids may also be involved. Responses controlled by photoperiods such as germination, flowering time, stem elongation, leaf abscission are all affected by brassinosteroids (Chory and Li, 1997), suggesting their role in controlling a plant's response to photoperiod.

### 1.5.3 Gravitropism and phototropism

Gravitropism is the directional growth of plant organs with respect to gravity. Primary roots often show positive gravitropism, i.e. they grow downwards; shoots normally show negative gravitropism, i.e. they grow upwards. The perception of gravity in roots is associated with amyloplast sedimentation in starch sheath cells around the phloem of shoots and in cells of the root cap. Movement of amyloplasts triggers other changes such as redistribution of auxin which in turn is thought to cause the growth changes that are associated with gravitropism (Hangarter, 1997). Roles for the second messengers such as $\text{Ca}^{2+}$ and calmodulin have also been implicated (Lu *et al.*, 1996). The interaction of statoliths (plastids (amyloplasts) containing starch grains) with the cytoskeleton is thought to transduce the perceived gravity signalling into a biochemical /physiological signal that initiates the growth response. Gravitropism of inflorescence stems and hypocotyls is genetically separable (Fukaki *et al.*, 1996), and both are distinct from root gravitropism. The shoot gravitropism loci SGR1 (or scarecrow, SCR (Scheres *et al.*, 1995)) and SGR2
are implicated in both hypocotyl and inflorescence gravitropism, whereas SGR3 is involved in hypocotyl gravitropism alone (Fukaki et al, 1996). SCR and SHR (short root) are required for normal endodermis formation in shoots and roots (Fukaki et al, 1998). The endodermis contains sedimted amyloplasts for determination of gravitropic orientation.

The response to the gravity vector can be affected by other factors such as light, which leads to a role for phytochrome. Arabidopsis exhibits a clear change in gravitropism in response to light. Dark-grown hypocotyls show negative gravitropism, but hypocotyls exposed to R display a random orientation of growth. In Arabidopsis both phyA and phyB play a role in the effect of light on gravitropism; phyB controls gravitropism in a R/FR reversible manner, while phyA acts through a non-reversible VLFR. No other phytochromes are involved (Robson and Smith, 1996).

Gravitropism of roots can also be affected by light. The roots of the maize variety Merit show an enhanced gravitropism in response to R; this is modulated by phytochrome with a Ca^{2+}/calmodulin-dependent protein kinase homologue suggested to play a role (Lu et al, 1996).

The role of phototropism in young seedlings is important to allow adaptation for maximum capture of light for photosynthesis. In older plants the role of phototropism is mainly restricted to leaf movements. Phototropism and gravitropism are distinct responses but their pathways share certain features; for example, auxin redistribution is implicated in both.

A photoreceptor encoded by NPH1, which is capable of absorbing B, UV-A and green light, is thought to be required for all the phototropic responses of Arabidopsis (Liscum and Briggs, 1995). NPH1 is a 120 kDa phosphoprotein photoreceptor (Liscum and Briggs, 1996).
Both phyA and phyB are involved in phototropism of Arabidopsis. Phototropism is the directional growth towards B. Phototropism occurs in two phases; first positive phototropism obeys the reciprocity law and is induced by low fluence rates of short time period; second positive phototropism occurs in response to high fluence rates of longer duration, and does not obey the reciprocity law. First positive phototropism, in response to R of a very low or low fluence rate, is enhanced by phyA. A large increase in seedling curvature arises when a pulse of R is given before the phototropic B light (Parks et al, 1997). The high fluence range has also been shown to enhance first positive phototropism but this is not a response of phyA or phyB. The enhancement of second positive curvature also requires phytochrome; phyB is responsible for most of this response to R. Analysis of phytochrome mutants implicates the role of another phytochrome in R (Janoudi et al, 1997). B light activation of phyA is also involved in second positive photocurvature. Gravitropism of hypocotyls is normal under W so phototropism caused by B probably counteracts the randomisation caused by R.

The relationship between phytochromes and gravitropism will be investigated in this thesis by analysis of an Arabidopsis mutant which has reduced hypocotyl randomisation under R and FR.

1.6 Dissection of phytochrome function and signalling pathways

All of the information perceived by the different photoreceptors combined together leads to overall morphogenic changes of the plant. There are a number of approaches which can be taken to identify components of signal transduction pathways and to determine their function. One approach to determine the functions of each individual photoreceptor is to express the gene for that photoreceptor differently to observe any changes in morphology induced. This can be achieved by mutation of the gene so the photoreceptor is inactive or has altered function/expression. Alternatively, overexpression of a particular phytochrome can be achieved by its insertion into a plant under the control of a known promoter leading to amplification of the response controlled by that photoreceptor.
Both of these approaches have been used, and are enabling the allocation of various functions to individual or multiple phytochromes, and are also leading to the dissection of the pathways involved in the transmission of the light signal to cause a developmental response.

Other methods recently yielding success in the analysis of phytochrome signalling include yeast two-hybrid screening and fluorescent differential display. The identification of PIF3 was achieved by utilising the yeast two-hybrid system with the phyB C-terminal domain as bait (Ni et al., 1998, 1999). PKS1 was identified through yeast two-hybrid studies, and was found to interact with the C-terminal domains of both phyA and phyB (Fankhauser et al., 1999). Using the phyA C-terminal domain of phyA as bait in the yeast two-hybrid screen also identified nucleoside diphosphate kinase 2 (NDPK2). NDPK2 is thought to be involved in the phyA-mediated FR HIR, and also in the phyB-mediated R HIR (Choi et al., 1999). The fluorescence differential display technique has been used to identify 15 phyA regulated genes (Kuno et al., 2000); however, all of these were also regulated by other phytochromes and so are probably end points of phytochrome signalling pathways.

1.6.1 Seedling morphology

The selection and characterisation of photomorphogenic mutants continues to reveal important information on the transduction of light signals, and this thesis is based upon that approach. To use mutant analysis or a transgenic approach, for altered seedling phenotype, in order to identify roles of photoreceptors and signalling components, it is first necessary to characterise the phenotype of wild type plants under different conditions. Seedlings grown in darkness have undeveloped chloroplasts, elongated hypocotyls, and their cotyledons remain closed and hooked over; this presumably serves to protect the meristem during growth through the soil. This developmental process is called etiolation or skotomorphogenesis and the process of de-etiolation or photomorphogenesis is triggered by light. During de-
etiolation hypocotyl extension is inhibited and resources are transferred to the opening and expansion of the leaves.

Plants grown under any continuous light condition (W, B, R or FR) show inhibited hypocotyl elongation and stimulated expansion of cotyledons. Under continuous FR the cotyledons remain pale because short wavelength energy (R) is required for action of protochlorophyllide reductase (por), the enzyme which reduces protochlorophyllide to chlorophyllide; this is the only light dependent step in the synthesis of chlorophyll. Transcription of chloroplast-encoded genes and plastid DNA replication can still occur under continuous FR, so phyA can mediate some steps of plastid development.

1.6.2 Chromophore-deficient mutants
The mutants hyl and hy2 in Arabidopsis both show reduced sensitivity to light; however the wild type phenotype can be rescued in the Arabidopsis mutants hyl and hy2 by application of exogenous biliverdin-IXα which is a precursor of the chromophore (Quail, 1991). So, these mutations occur in the chromophore biosynthesis pathway rather than in a phytochrome gene itself or in a gene encoding a component of a signal transduction pathway controlled by phytochrome.

A number of chromophore-deficient mutants have been isolated other than those above; the au (aurea) and yg-2 (yellow-green) mutants of tomato (Koornneef et al, 1985), pcd1 and pcd2 (phytochrome chromophore deficient) mutant of pea (Weller et al, 1996, 1997) and three pew (partially etiolated in white) loci in Nicotiana plumbaginifolia (Kraepiel et al, 1992). Phytochrome chromophore mutants have in common a number of phenotypes; they are tall under W, R and FR, they have increased apical dominance, retarded leaf development and defects in chloroplast development resulting in pale/yellow leaves (severity dependent upon growing conditions). The number of chloroplasts is reduced with each having less granal stacking, resulting in less chlorophyll. An increase in the ratio of chlorophyll a to chlorophyll b occurs, this can be explained as the chloroplasts in these mutants do
not develop fully from the proplastids and therefore contain higher levels of chlorophyll a (Chory et al, 1989). Despite the deficiency of chromophore, PHYA (the apoprotein) still accumulates in the dark, although the extent depends upon the plant species (Kraepiel et al, 1992, Weller et al, 1996). PHYB levels are unaffected (Weller et al, 1996).

PΦB is synthesised via the haem branch of the tetrapyrrole pathway from 5-aminolevulinic acid (ALA), in the plastid. Mature au plants have a normal photosynthetic rate, and generally chromophore-deficient plants are healthy and vigorous, which suggests that these mutants are blocked after haem (Terry 1997). The next step in PΦB synthesis following haem is the conversion by haem oxygenase to biliverdin. As the hy1 and hy2 mutants of Arabidopsis can be rescued by supplementation with biliverdin these mutants are likely to lack haem oxygenase activity.

The phytochrome responses of chromophore-deficient mutants recover as the plants mature. Mature au plants show wild type shade avoidance and EOD responses, and the pcd1 mutant responds more to EOD FR as it ages. Two explanations are proposed by Terry (1997) for these observations; the mutations could be leaky, so chromophore could accumulate in light-stable phytochromes to recover these responses, or there may be additional genes encoding chromophore biosynthesis enzymes, the expression of which could increase during development of the plant.

Two suppressors of the hy2 mutation have been isolated, shyl-1D and shy2-1D. These are dominant mutants which show many photomorphogenic characteristics also in the dark (Kim et al, 1998). These mutations define two novel components of Arabidopsis photomorphogenesis. SHY1 is involved with the phyB, red-light-mediated inhibition of hypocotyl elongation, whereas SHY2 is involved with both R and FR inhibition of hypocotyl elongation. Although these mutants show light-grown phenotypes in the dark they are distinct from cop and det, which are recessive
mutations and do not show the abnormal leaf development of shy1 and shy2. So shy1 and shy2 are extragenic dominant mutations and not true revertants of hy2.

1.6.3 Phytochrome mutants
A number of phytochrome mutants have been isolated. In Arabidopsis thaliana phytochrome A, B, D and E mutants are well known. Mutants that are deficient in individual photoreceptors have also been isolated in some other species such as tomato, cucumber, tobacco, Sorghum and pea.

1.6.3.1 Phytochrome A
Phytochrome A is the only "light-labile" phytochrome; relatively high levels accumulate in the dark, but the pool is rapidly depleted in light, although a very low residual level remains. In etiolated seedlings up to 99% of the total phytochrome pool can be phyA, which can constitute up to 1% of the total etiolated seedling protein (Clough and Vierstra, 1997).

Phytochrome A mutants show wild type phenotypes in R, W and D but display the dark-grown etiolated phenotype when grown in continuous FR. The fri (far-red insensitive) mutant of tomato is equivalent to phyA in Arabidopsis and it displays the same absence of response to FR with reduced inhibition of hypocotyl elongation.

The high abundance of phyA in etiolated tissues indicates its likely role in greening and de-etiolation. Phytochrome A is responsible for the FR-HIR (accumulation of anthocyanin, inhibition of hypocotyl elongation, and stimulation of cotyledon opening in Arabidopsis), demonstrated by the normal action of this response in phyB and the isolation of mutants which lack detectable phyA and selectively have no FR-HIR (Nagatani et al, 1993, Whitelam et al, 1993).

In the natural environment the main role of phyA in a wild type plant is to control the initial de-etiolation of the seedling as it emerges from the soil, although roles in daylength perception and control of gene expression also exist. Arabidopsis phyA
mutants grown under natural canopy shade have extremely long hypocotyls and the de-etiolation response is impaired (Yanovsky et al, 1995). The hypocotyls of phyA mutants are elongated compared to wild type, following growth in low R:FR ratios (Whitelam and Devlin, 1997). phyA is thought to inhibit the extension growth of emerging seedlings to prevent shade-avoidance responses predominantly mediated by phyB under low R:FR ratios (Smith et al, 1997). The double mutant phyAphyB is longer under low R:FR ratios than the phyB mutant alone, suggesting phyA normally inhibits hypocotyl elongation in the phyB mutant. So in the natural environment under shade conditions phyA antagonises the elongation caused by predominantly phyB detection of low R:FR ratio, thus preventing a seedling from exhausting its resources. Mutants which lack phyA show a high seedling mortality under natural shade conditions/low R:FR ratios, this suggests that phyA controls the degree of shade-avoidance in small seedlings (Yanovsky et al, 1995). The extent of the response of phyB to reduced R:FR ratios is modulated by phyA (Casal et al, 1997); overexpression of phyA in tobacco leads to the lack of early neighbour detection (McCormac et al, 1991, Casal and Sánchez, 1994). Phytochrome A deficient mutants have longer hypocotyls than wt when grown under light/dark cycles; however, under continuous white light the hypocotyls are of the same length. The dark cycle may allow time for the light labile phytochrome to reaccumulate in the wt and therefore have an active effect in the next light period.

phyA acts with phyB to maintain the rosette habit of Arabidopsis. In response to EOD FR the phyAphyB double mutant shows internode elongation within the rosette; this is not observed in the wild-type or phyB mutant (Johnson et al, 1994).

The germination of Arabidopsis in response to FR has been shown to be mediated by phyA (Johnson et al, 1994). Taking seeds of wild type (wt), phyA and fhy1 (a phytochrome A signalling mutant) which do not germinate in D and exposing them to FR or R demonstrated that phyA was required for germination in FR but not in R.
Experiments on flowering time of Arabidopsis have shown that phyA is active in mature plants. Under short day (SD) conditions (8 h W, 16 h dark) wt, phyA and *fhy1* all flower at about the same time, however if these SD are extended (8 h W, 8 h low fluence incandescent light, 8h dark) then phyA is late flowering; under long days (LD) phyA flowers at almost the same time as wt (Johnson et al, 1994).

Phytochrome A plays a role in the agravitropism of hypocotyls through a VLFR (section 1.5.3, Robson and Smith, 1996). phyA could also be involved in the transition from skotomorphogenesis to photomorphogenesis, by regulating assimilate translocation, suggested by the localisation of *PHYA* promoter activity in specialised phloem cells of Arabidopsis and tobacco (Adam et al, 1997).

1.6.3.2 Phytochrome A signal transduction mutants
A number mutants have been isolated which identify phyA specific signal components. These include the Arabidopsis mutants *fhy1* and *fhy3* (far-red elongated hypocotyl); hook opening and cotyledon expansion is not stimulated by FR in these mutants (Whitelam et al, 1993). The *far1* mutant is specific to phyA signalling, and shows reduced inhibition of hypocotyl elongation at high fluence rates of FR (Hudson et al, 1999). Isolation of suppressors of mutations allows identification of new signal transduction intermediates. There is one reported example of a suppressor within the phytochrome A pathway; *SPA1* suppresses a weak *phyA* (*phyA105*) mutant phenotype of Arabidopsis RLD ecotype (Hoecker et al, 1998). phyA specific signal transduction is discussed in more detail in section 1.7.

1.6.3.3 Phytochrome B
Mutants that are deficient in phytochrome B (e.g. *hy3* (*phyB*)) in Arabidopsis, *lh* (*long hypocotyl*) in cucumber, *ein* (*elongated internode*) in *Brassica rapa*, *tri* (*temporarily red insensitive*) in tomato, and *lv* in pea) have an etiolated phenotype with reduced cotyledon/leaf expansion and altered anthocyanin levels under W or R, but they show normal wild type characteristics under FR. In Arabidopsis *phyB* there
are fewer chloroplasts so the greening process is affected. The mutant adult plants show increased apical dominance and increased stem and petiole elongation. The shade-avoidance and EOD responses are reduced. Wild type plants grown under day/night cycles show an elongated stem or hypocotyl response compared to wt if the end of a photoperiod is followed by a pulse of FR. This is the EOD response. Elongation growth mediated by EOD FR is primarily due to phyB. In wild type plants the stable PfrB formed during the day continues to act through the night inhibiting hypocotyl elongation; EOD FR removes most of this PfrB and therefore the hypocotyls elongate. An elongation response can still be observed in phyB as can an early flowering response to EOD FR, so other phytochromes are implicated in these responses (Whitelam and Smith, 1991). The tomato PHYB1 mutant is comparable to the Arabidopsis phyB but these plants do display some slightly different responses; they are not affected in the EOD-FR response, and are only temporarily insensitive to R (Pratt et al, 1997). Under high R:FR ratios the phyB mutant of Arabidopsis is elongated, and shade avoidance responses to low R:FR ratios are reduced although not completely absent (Whitelam and Smith, 1991). Light grown phyB mutants show a similar phenotype to wild type plants grown under low R:FR. Early flowering of phyB is observed under long day and short day conditions and also under high R:FR ratios. However, growth under low R:FR ratios still causes a promotion of flowering and therefore shade avoidance involves other phytochromes (Whitelam and Smith, 1991, Robson et al, 1993).

Germination of wild type or phyA Arabidopsis seeds can be promoted by a pulse of R light; in phyB mutants this response is much reduced; phyB mutants also show low germination frequencies in the dark. It is proposed that PfrB plays a role in the germination of Arabidopsis seeds (Shinomura et al, 1994). Higher germination frequencies can be obtained in phyB mutants than in wild type following continuous FR irradiation (Shinomura et al, 1994). A possible explanation for this may be that in the wild type any PfrB present will be converted to PrB, suppressing germination. At the same time continuous FR will induce the action of phyA which promotes seed germination, so it is proposed that PrB, although normally the inactive form,
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retains some residual activity and can antagonise this phyA promotion of germination (Shinomura et al, 1994). Other photoreceptors are involved in the germination of seeds, as the phyAphyB double mutant still shows promotion of germination in response to continuous, or multiple pulses of R, and retains a R/FR reversible promotion of germination (Whitelam and Devlin, 1997).

The hlg (hypocotyl long) mutants of Nicotiana plumbaginifolia have wild type levels of PHYB mRNA, but no detectable PHYB apoprotein. These mutants show a reduced inhibition of seedling elongation in R, and have a wild type phenotype under other monochromatic light wavelengths, as observed in other phyB mutants (Hudson et al, 1997). However, their adult phenotype is different to that of other phyB mutants. The hlg mutants are late flowering under LD and, unlike wild type, bolting is not delayed under SD (Hudson and Smith, 1998). The rhythm of leaf movement in hlg-1 lacks sensitivity to photoperiod, the movement being constitutively that of a SD entrained plant. It is proposed that phyB enables the endogenous oscillator to determine daylength, but the leaf movement rhythm is entrained by another photoreceptor (Hudson and Smith, 1998). The fact that phyB mutants of different species have different phenotypes suggests that the analysis of Arabidopsis as a model species may be misleading. The role of phytochromes and signal pathway components may vary between species.

Phytochromes A and B show some redundancy. For example, promotion of cotyledon expansion and induction of CAB gene expression in response to R involves both phyA and phyB. In the absence of one of these phytochromes the response is little affected so the remaining phytochrome can carry out the complete response; however, in the absence of both phytochromes the response is severely affected. Other examples of redundancy between phyA and phyB include the hypocotyl elongation response to R, and maintenance of normal biomass production under 10h light, 14h dark photoperiods (Devlin et al, 1996).
Phytochromes A and B also act synergistically; a single pulse of R even when given every day leads to only very little cotyledon unfolding and inhibition of hypocotyl elongation. However, a pre-treatment of continuous FR which is perceived by phyA enhances the response to a terminal R versus FR pulse perceived by phyB (Casal et al., 1997). The transgenic phyA mutant displays an exaggerated LFR for expression of Lhcb1*2gusA under R pulses (Cérdat et al., 1999); this suggests a negative regulation of phyB activity by phyA. phyA also mediates a positive effect on phyB-mediated responses; the LFR induced by a R pulse is enhanced by a FR pre-treatment perceived by phyA (Cérdat et al., 1999).

### 1.6.3.4 Phytochrome B signal transduction mutants

*red1* is a phyB specific signal transduction mutant (Wagner et al., 1997). This mutant has elongated hypocotyls, smaller cotyledons, elongated petioles and reduced shade avoidance, characteristic of a phyB mutant, but the *RED1* locus maps to a different chromosome location. It is suggested that *RED1, FHY1* and *FHY3* may be genes encoding phytochrome reaction partners (Wagner et al., 1997). These could have domains for specific recognition of phyA or phyB, and a second domain for interaction with the proposed regulatory C-terminal domain (Xu et al., 1995). Alternatively, *RED1, FHY1* and *FHY3* could encode interaction partners which selectively bind to phyA or phyB N-terminus, with a second non-selective partner binding afterwards to the C-terminus (Wagner et al., 1997). The mutants *pef2* (phytochrome-signalling early flowering 2) and *pef3*, may also be phyB specific signal transduction mutants (Ahmad and Cashmore, 1996). These mutants show phenotypes which are characteristically associated with phyB lesions such as early flowering and long hypocotyl under R. The *pef1* mutant of Arabidopsis is insensitive to both R and FR and therefore defines a component of both the phyA and phyB signalling pathways. One mutant (*psi*-phytochrome signalling) has been isolated which is hypersensitive to both R and FR. It is proposed that *psi2* may also act downstream of both phyA and phyB (Genoud et al., 1998).
The pod (photocurrent 1) mutant of Arabidopsis has enhanced phyB signalling, this is caused by the insertion of a T-DNA in the promoter region of PIF3 (phytochrome interacting factor 3) leading to overexpression of this gene (Halliday et al, 1999b). The chlorate-resistance mutant, cr88 (Lin and Cheng, 1997), may also define a component of phyB signalling. This mutant displays a long hypocotyl under R, suggesting reduced phyB signalling, but also displays delayed greening, and has reduced expression of the light-induced NR2 gene. It is possible that CR88 regulates NR and greening via the phyB pathway, in a similar way to phyA-mediated FHY1 regulation of these responses under FR.

1.6.3.5 Other phytochromes

Phytochromes C, D and E are less well characterised. Some accessions of the naturally occurring Arabidopsis ecotype Wassilewskija (Ws) contain a deletion in the PHYD gene (Aukerman et al, 1997). As this plant is found in the wild the lack of phyD cannot confer a significant selective disadvantage, at least in its native environmental conditions. The mutation in Ws is caused by a 14 bp deletion which causes early truncation of the phyD apoprotein (Aukerman et al, 1997). The phenotypes observed as a result of the phyD mutation, such as a slightly elongated hypocotyl and slightly reduced cotyledon expansion under R, reduced anthocyanin accumulation, reduced EOD-FR response and early flowering, are all similarly observed in the phyB mutant. Some of these responses are additive, such as control of hypocotyl length, although phyB plays the major role. The double mutant phyAphyB still shows a R/FR reversible response for early flowering and internode elongation; phyD plays a role in this remaining activity. The phyBphyD double mutant still displays an EOD-FR response so other phytochrome(s) must be involved. Like phyB, phyD has been shown to interact with CRY1 for inhibition of hypocotyl elongation under R pulses (Hennig et al, 1999). phyD and phyB are the result of a fairly recent gene duplication, so it is not surprising that they display some similar functions.
A phyE mutant of Arabidopsis has been isolated (Devlin et al, 1998). As a monogenic mutation it is indistinguishable from the wild type. However, the phyA phyB phyE triple mutant is constitutively early flowering and has internodes between rosette leaves characteristic of the phyA phyB double mutant under EOD-FR. Early flowering of phyE is only observed in a phyB background. So phyB, phyD and phyE show some redundancy for inhibition of flowering. Monogenic phyE and the double mutants phyA phyE and phyB phyE have a rosette habit; elongated internodes are only observed in the phyA phyB phyE mutant, so inhibition of internode elongation can be suppressed by the action of any one of these phytochromes.

1.6.3.7 COP and DET
At least ten mutants (Torii and Deng, 1997) have been isolated which when grown in the dark show characteristics of light-grown plants. These include det (de-etiolated) mutants, cop (constitutively photomorphogenic) mutants, and fus (fusca) mutants. The det and cop mutants were isolated in screens to look for light-grown phenotypes in dark-grown plants. The fus mutants were isolated because of their high anthocyanin accumulation giving them a purple seed colour; these are lethal after the seedling stage. The DET/COP/FUS genes appear to lie downstream of known photoreceptors. Some encode proteins involved in steroid hormone biosynthesis (e.g. DET2), and others encode nuclear proteins (e.g. COP1, COP9 and DET1) which may be negative regulators of gene expression (Chory et al, 1996). Their negative regulatory function is implied by the fact that these mutations are recessive so the wild-type genes are repressing photomorphogenesis in the dark (Torii and Deng, 1997). The phenotypes of these mutants are similar suggesting that they affect the primary change from skotomorphogenesis to photomorphogenesis, before any major pathway branches to control for example plastid development or hypocotyl elongation. This hypothesis is supported by the suppression of photomorphogenesis in B and FR using overexpression of COP1 in Arabidopsis (McNellis and Deng, 1995). Transition to photomorphogenesis is a complex process involving the mobilisation and translocation of reserves. Other hypotheses exist to
explain the related phenotypes of the *det/cop/fus*. They might occur in multiple parallel pathways controlling onset of photomorphogenesis or the proteins may function in close proximity in the same pathway.

The COP1 protein contains a number of well-characterised domains including an N-terminal zinc-binding RING-finger, a predicted coiled coil and C-terminal WD-40 repeats, these occur in a novel combination (Torii and Deng, 1997). It is proposed that the nuclear abundance and therefore activity of COP1 is controlled by light, being located in the nucleus in the dark and the cytoplasm in the light. The probable role of COP1 in repression of gene expression is supported by its structure. Cellular and biochemical data also suggest that the COP9 complex could regulate transcription as the complex of COP9 and FUS6 is located in the nucleus (Chamovitz and Deng, 1997). It is proposed that COP1 and COP9 interact during the dark to localise COP1 in the nucleus. More recently it has been shown that COP1 and HY5 interact in the nucleus (Ang et al, 1998). It is proposed that COP1 interacts in the dark with transcription factors such as HY5, inactivating their transcriptional activity. The reduced nuclear abundance of COP1 in the light allows transcription to proceed (Jarillo and Cashmore, 1998). Analysis of GUS-COP1 in photoreceptor mutants has revealed that the photoreceptors co-operatively regulate the GUS-COP1 localisation. In FR, R and B the localisation of GUS-COP1 is regulated by phyA, phyB and CRY1 respectively. There are also overlapping roles, phyA influences B localisation of COP1 and there are indications that CRY1 may influence COP1 localisation under FR (Osterlund and Deng, 1998). This may occur if CRY1 has a synergistic action with phyA in FR.

1.6.4 Phytochrome overexpression studies

1.6.4.1 Phytochrome A

Overexpression of the PHYA gene in for example Arabidopsis, tomato and tobacco leads to amplified responses to FR and W and in some cases to R. It is not expected that phyA plays a significant role in R perception by wild type plants because phyA concentration is normally depleted under such conditions, so the response in the
transgenic plants is due to the constitutive ectopic expression of the apoprotein. This suggests that either the high levels of phyA can trigger the R light signal transduction pathway or the ectopic expression of one phytochrome can perform the function of another endogenous phytochrome assuming the downstream components of the pathway are shared (McNellis and Deng, 1995). Mazzella et al (1997) demonstrated that the enhanced response to R due to overexpressed phytochrome A is due to a VLFR which is occurring at R fluence rates below $10^{-2} \mu\text{mol m}^{-2}\text{s}^{-1}$.

Overexpression of oat or rice phyA in tobacco or Arabidopsis wt and phyB enhances R and W mediated inhibition of hypocotyl elongation (McCormac et al, 1991, Halliday et al, 1999a). The reduced sensitivity of phyB to R is corrected for some responses in Arabidopsis overexpressing rice phyA; these results show that phyA plays a role in R to control some of the same responses as phyB. However, there is no increased sensitivity to FR in the phyB mutant overexpressing the rice phyA and only a small increase in hypocotyl inhibition in the wild type overexpressing lines (Halliday et al, 1999a). This is different to the overexpression of oat phyA in Arabidopsis (McCormac et al, 1991) where enhanced sensitivity to both R and FR is observed. So the two cereal phytochromes may act differently for the FR HIR when overexpressed in a dicotyledonous plant. Overexpression of the rice phyA in the Arabidopsis phyB mutant is unable to restore responses to low R:FR ratio and EOD FR (Halliday et al, 1999a). The FR-HIR is only transient in wild-type plants as the phytochrome A levels deplete, but transgenic Arabidopsis and tobacco which express 35S:PHYA retain the FR-HIR after de-etiolation (McCormac et al, 1992).

Experiments by Rousseaux et al (1997) have shown that regulating PHYA overexpression to specific regions of the plant allows a reduced responsivity to FR in some tissues without changing normal phytochrome responses in the rest of the plant. Tobacco overexpressing high levels of CAB:PHYA in the apex and leaves and low levels in the stems do not respond to FR directed to the leaves but increase stem length significantly if FR is directed to the whole shoot or specifically to the internodes.
1.6.4.2 Phytochrome B

Plants overexpressing *PHYB* are early flowering, which considering the early flowering of the *phyB* mutant is surprising. Other phenotypes observed in Arabidopsis overexpressing *PHYB* include smaller cotyledons, shorter hypocotyls, reduced petiole and leaf length and reduced apical dominance (Wagner *et al*, 1997). Antisense *PHYB* in potato leads to a loss of photoperiodic control of tuberization (Jackson *et al*, 1996).

Transgenic plants overexpressing *PHYB* cDNA have high germination frequencies in the dark. The frequency is not affected by the R:FR ratio under which the parent plants are grown, whereas wild type seeds show lower dark germination and are strongly influenced by the R:FR ratio the parental plants are grown under (McCormac *et al*, 1993). These results may correlate with the levels of phyB Pfr indicating a direct role for phyB in germination of Arabidopsis seeds.

1.6.4.3 Phytochrome C

Transgenic tobacco overexpressing Arabidopsis phyC show a R-dependent increase in cotyledon expansion and the mature plants have increased leaf area, stem length and girth (Halliday *et al*, 1997). Overexpression of Arabidopsis phyC in Arabidopsis leads to a R-dependent enhancement of hypocotyl elongation inhibition, with no change in the effect of FR upon hypocotyl inhibition. An increase in primary leaf expansion is observed under continuous W (Qin *et al*, 1997), and transgenic plants overexpressing phyA or phyB do not have enhanced primary leaf expansion. So phyC appears to be similar to phyB in terms of photosensory ability; however, it may have physiological roles distinct from phyA and phyB.

1.7 Focus on phyA signalling

A number of mutants have been isolated that define components of the phyA signalling pathway. The Arabidopsis mutants *fhy1* and *fhy3* show reduced sensitivity to FR. The spectrally active phytochrome of these mutants appears to be no different to wt, and wt levels of immunochemically detectable phyA are present. Both of
these mutations are recessive (Whitelam et al, 1993). Stimulation of germination of \textit{fhiyl} by FR is unaffected so only certain phyA responses are altered in this mutant (Johnson et al, 1994). FHY1 and FHY3 therefore encode phytochrome A signal transduction pathway components.

To investigate the possible role of FHY1 in phyA signalling, chalcone synthase (\textit{CHS}) and \textit{CAB} mRNA levels were measured; these are products affected by the phytochrome A signal transduction pathway (Barnes et al, 1996a). From these experiments it was proposed that \textit{fhiyl} defines a branch point in the phytochrome A signal transduction pathway as induction of nitrate reductase (\textit{NR}) and \textit{CAB} still occurs, whereas induction of \textit{CHS}, \textit{CHI} (chalcone isomerase), \textit{PC} (plastocyanin), and \textit{RBCS} (rubisco) is reduced. The induction of \textit{CHS} occurs via the cGMP branch of the phytochrome A signal transduction pathway, so FHY1 probably participates in this branch. FHY1 has been more recently shown to be necessary for the interaction of the phyA pathway with the phyB pathway (Cerdán et al, 1999).

The \textit{far1} mutant is specific to phyA signalling and shows reduced inhibition of hypocotyl elongation at high fluence rates of FR (Hudson et al, 1999). Three genes with high homology to \textit{FAR1} have been identified using Blast searches, but \textit{FAR1} shows no homology to any proteins of known function. \textit{FAR1} does however, have a functional nuclear localisation signal and has a predicted coiled-coil region, which supports a role for \textit{FAR1} in protein-protein interactions, as certain other light signalling components such as SPA1 and COP1 also have predicted coiled-coil regions.

\textit{spal}, isolated as a suppressor of \textit{phyA}, is a recessive mutation suggesting SPA1 is a negative phyA signal transduction regulator. SPA1 is a novel nuclear protein with a WD repeat domain in the C-terminal end which is thought to be involved in protein-protein interactions (Hoecker et al, 1999). This WD repeat domain shows high homology to that of COP1 and is essential for SPA1 activity.
1.8 Thesis outline

Although more is known about the phyA signalling pathway than other phytochrome pathways there is still much to be resolved. As more components are discovered the pathway becomes more complex. In this thesis three approaches to increasing the knowledge of the phyA pathway and its interaction with the phyB pathway and other pathways regulating plant morphology are presented. Interaction between phytochrome signalling and gravity perception is investigated by analysis of a mutant that is gravitropic in light (gil). Physiological characterisation of this mutant suggests that a branch point in phyA and phyB regulation of responses to the gravitational vector is defined. Molecular analysis reveals that the disrupted gene that most likely mediates the gil phenotype has no homology to existing genes.

Characterisation of the FR and R light responses of fhy3, suggests that FHY3 plays a role in both phyA and phyB-mediated de-etiolation. Isolation of suppressors of fhyl is described. These mutants display suppression of some fhyl phenotypes and may define branch points in phyA signalling.
2.0 Materials and Methods

2.1 Plants and growing conditions

2.1.1 Seed sterilisation

Seeds were sterilised firstly with 70% ethanol for 1 minute, followed by 10% sodium hypochlorite with a few drops of Tween for 5-10 minutes. The seeds were then washed three times with sterile distilled water, placed on sterile filter paper and allowed to dry.

2.1.2 Seed germination

Seeds for experiments where synchronous germination was required were placed onto agar plates (2.2.1.1). The plates were immediately wrapped in foil and placed at 4°C for 3d. Plates were exposed to a 1h white light pulse (40 μmol m⁻² s⁻¹) to induce germination of the seeds, this was followed by return of the plates to darkness for a further 24h at 25°C. Plates were then subjected to the appropriate experimental conditions.

2.1.3 Growing adult plants

Seeds were germinated on agar plates, and after approximately 2 weeks were transferred to a mixture of compost, sharp sand and vermeipearl (graded horticultural vermiculite).

Adult plants were grown in a heated greenhouse (21°C) with supplementary light, for LD non-experimental purposes. Controlled long day and short day experiments were carried out in Fisons cabinets, the temperature and daylength can be regulated in these cabinets.

2.1.4 Crossing of Arabidopsis

Arabidopsis plants were grown to flowering in the greenhouse. Siliques and any open flowers were removed from the inflorescence stem to be used. The central meristem was removed under a binocular microscope to leave the 4 largest
unopened buds. These buds were opened with fine tweezers and all anthers were removed. Anthers from the donating plant were used to transfer pollen onto the stigma. The sepals of the bud were gently re-closed and the bud was wrapped in clingfilm for a few days to prevent drying. Clingfilm was then removed and the siliques allowed to develop. Seed was harvested into paper bags from the individual siliques once they had matured. Seed was allowed to dry for several weeks before use.

2.1.5 Monochromatic light sources

Unless otherwise stated, seedlings were grown under maximum irradiance at the base of the appropriate cabinet, for spectral scans see appendix 2. The red light source consisted of light emitting diodes (LED). Experiments requiring R and FR together were carried out under a mixed array of R and FR LED, these were obtained from Quantum Devices, Inc., Barneveld, WI, USA. Continuous FR was achieved by selecting only FR LEDs of the mixed array, or by fluorescent tubes (supplier??). Unless otherwise stated experiments in which seedlings were exposed to monochromatic light proceeded over 3d, under fluence rates: $\sim 15 \mu \text{mol m}^{-2} \text{s}^{-1}$ R, $\sim 30 \mu \text{mol m}^{-2} \text{s}^{-1}$ FR.

Measurements of irradiance were made using a hand held LI-COR photometer, model LI-189 for measurement of R, and a portable LI-COR, model LI-1800 for measurement of FR and R/FR ratios.

2.1.6 Fluence rate experiments under monochromatic light

Seeds were germinated on 5 cm petri-dishes, these were transferred in to black plastic boxes with clear lids. The position of these boxes within the cabinets, and the number of layers of Whatmann 3MM paper or muslin placed on top of the boxes was adjusted to provide the required fluence rate.
2.1.7 Physiological measurements of Arabidopsis

Seedlings were laid onto agarose plates and photographed using Fuji Provia 100 colour slide film. The processed film was projected onto a Wacom digitising tablet and traced on the tablet into Sigma-Scan software. Hypocotyl lengths, cotyledon angles and cotyledon areas were measured in this way. Errors were calculated as value for 95% confidence interval \((x \pm t(v,z) (s/\sqrt{n}))\) where \(x\) is mean, \(s\) is standard deviation, \(t(v,z)\) the statistic for \(v=n-1\) degrees of freedom and \(z=1.96\) standard normal percentile equivalent.

2.1.7.1 Screening for mutants with altered gravitropic responses to light

Seeds were placed onto square petri-dishes and stored at 4°C in the dark for 4d in a horizontal position. Plates were then subjected to a 3h W light pulse, and returned to 4°C dark in a vertical position. After 24h the plates, in the same orientation, were transferred to 25°C dark. 12h later, when the seedlings had reached 3-5 mm in length, the plates were returned to the horizontal position and subjected to screening conditions, 5 min FR every 30 min. After 12h in the screen conditions the plates were rotated a further 90° to the vertical position (180° rotated from the previous vertical position). Candidate mutants were selected 12h later. The seedlings selected would be those which displayed a "U-shaped" hypocotyl, i.e. hypocotyls which had followed the rotation of the plate (figure 2.1).

Figure 2.1 Plate rotation screen. Seedlings which do not randomise in light will have a characteristic "U-shape".


2.1.7.2 Determination of the degree of randomisation

Seeds of wild type and gill were placed on a square agar plate, arranged as one horizontal line of 30 seeds for each. These plates were stored in a vertical position perpendicular to the rows of seeds. The procedure for germination was followed as reported in section 2.1.2, the plates remained vertical at all times. Following the final 24h D, 25°C of the germination procedure, the plates were either maintained in darkness or transferred to the appropriate light treatment for 3d. The plates were positioned slightly offset from the vertical to prevent the seedlings from growing along the agar which could affect their orientation. After 3d the plates were photographed. Randomisation was measured as the angle between the hypocotyl and the vertical, so a seedling that grows straight upwards will have an angle of 0°. Positive numbers were assigned to those hypocotyls bending to the right and negative numbers to those bending to the left. To measure the angle of hypocotyl growth, the photographs were projected using a photographic enlarger onto a piece of paper with a horizontal line drawn on it. Hypocotyls were then traced onto the paper in their respective positions. The angles were measured to the nearest degree using a protractor.

2.1.8 Anthocyanin detection

Fifty seedlings were transferred to a 1.5 ml microcentrifuge tube containing 1 ml methanol containing 1% HCl. The tubes were shaken in the dark for 72 h. The amount of anthocyanin was calculated by the equation $A_{530} - 0.25 A_{657}$ (Mancinelli et al, 1991).

2.2 Media

2.2.1 Plant media

2.2.1.1 Lehle medium

Arabidopsis seeds were grown on Lehle medium; The following chemicals were added to 500 ml deionized water and stirred; 5 ml 1 M KNO$_3$; 2.5 ml 1 M KH$_2$PO$_4$; 2 ml MgSO$_4$; 2.5 ml Sequestrene (2.5 g FeSO$_4$-7H$_2$O in 400 ml H$_2$O, add 3.3 g NaEDTA, bring to boil, stir on magnetic stirrer for
30 min while cooling, make up to final volume 450 ml); 1 ml micronutrients (70 mM H₃BO₃, 14 mM MnCl₂·4H₂O, 0.5 mM CuSO₄·5H₂O, 1 mM ZnSO₄·7H₂O, 0.2 mM NaMoO₄, 0.01 mM CoCl₂). The volume was made up to 1 litre and divided into 500 ml flasks containing 3 g Agarose 15 (BDH). The medium was autoclaved and stored at room temperature.

2.2.1.2 Agarose plates

Plates for photographing seedlings were made from agarose (0.6%) dissolved in water using a microwave oven.

2.2.2 Media for bacterial growth

2.2.2.1 Luria broth (LB)

LB (Sigma) was made according to the manufacturers' instructions, and autoclaved before use.

2.2.2.2 Bacterial agar

Bacterial agar (LA) was made by the addition of 15 g/l Bio Agar (BG) to LB prior to autoclaving.

2.2.2.3 Top Agar

Top agar, used during library screening, was made in the same way as LA but 7 g/l Bio Agar was added. In the final stage of library screening, for growth of phage to be isolated, 7 g/l GibcoBRL electrophoresis grade agarose was added instead of agar.

2.2.2.4 Recombinant selection medium

Blue/white selection medium was made by the addition of; 400 µl ampicillin (1 g ampicillin dissolved in 5 ml H₂O + 5 ml ethanol, stored at -20°C); 400 µl Isopropylthio-β-D-galactoside (IPTG) (20 mg/ml); 400 µl 5-Bromo-4-chloro-3-
indolyl-β-D-galactoside (X-GAL) (20 mg/ml) to molten LB, plates were poured immediately in a flow hood.

2.3 Molecular biology techniques

2.3.1 Nucleic acid extraction

2.3.1.1 Extraction of plant genomic DNA

Two methods were employed for the extraction of plant genomic DNA, for small scale samples the Qiagen DNeasy minikits were used and for larger scale samples a method based on Doyle and Doyle (1987) was used.

2.3.1.1.1 Small scale extractions; Qiagen (tissue weight less than 100 mg)

See manufacturers' instructions for details of buffer contents.

All centrifugation was carried out in an Eppendorf microcentrifuge.

1) Plant tissue was frozen in liquid nitrogen and ground using a mortar and pestle. The cells were lysed with 400 µl buffer API, 4 µl RNase A (100 mg/ml) was added simultaneously.

2) The contents were then transferred to a microcentrifuge tube for vortexing. The tube was inverted 2-3 times during incubation at 65°C for 10 min.

3) Buffer AP2 (130 µl) was added and incubated on ice for 5 min, to precipitate proteins, polysaccharides and detergent.

4) Lysate was transferred to a QIAshredder spin column sitting in a 2 ml collection tube, and spun for 2 min at maximum speed.

5) The measured volume of flow-through was transferred to a new tube, 0.5 volumes of buffer AP3 and 1 volume of ethanol (EtOH) were added and mixed by pipetting.

6) Approximately 650 µl of the mixture was transferred to a DNeasy mini spin column sitting in a 2 ml collection tube and spun for 1 min at 6000 rpm. Flow-through was discarded.

7) The remaining sample was applied to the column and the procedure repeated.

8) The DNeasy spin column was transferred to a new collection tube and 500 µl buffer AW was applied.
9) The sample was spun at 6000 rpm for 1 min, flow through was discarded.
10) The column was spun for a further 2 min at maximum speed to dry the column membrane.
11) The column was transferred to a new collection tube and eluted by addition of 50 µl preheated (65°C) buffer AE, incubation at room temperature for 5 min and centrifugation at 6000 rpm for 1 min. The elution step was repeated and the two elutes were combined.

2.3.1.1.2 Large scale extractions; Doyle and Doyle (tissue weight 500 mg-1.5 g):
All centrifugation was carried out in a Beckman bench top centrifuge.
1) Leaf tissue was ground in a mortar and pestle with liquid nitrogen, and transferred to a 50 ml chloroform resistant tube.
2) Preheated (60°C) 2x CTAB isolation buffer (7.5 ml) (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% hexadecyltrimethylammonium bromide (CTAB), 0.2% 2-mercaptoethanol) was added to the still frozen leaf tissue and mixed. The tubes were incubated for 30 min at 60°C with occasional mixing.
3) Chloroform (7.5 ml) was added, mixed and the tubes spun at 3600 rpm for 2 min.
4) The aqueous phase was transferred to a new tube and 15 ml of cold isopropanol added. Samples were spun for 5 min at 1000 rpm.
5) Isopropanol was poured off and 20 ml wash buffer (70% ethanol, 10 mM ammonium acetate) added and incubated at room temperature for 20 min.
6) Samples were spun at 3600 rpm for 5 min, isopropanol poured off and the pellet air dried briefly.
7) The pellet was resuspended in 1 ml sterile water, and 1 µl RNaseA (final concentration 10 µg/ml) added. Samples were incubated at 37°C for 30 min.
8) H₂O (2 ml), 1.3 ml ammonium acetate (7.5 M), and 15 ml cold ethanol were added to the samples with gently mixing to precipitate the DNA.
9) Samples were spun at 10g for 10 min in a refrigerated centrifuge (Eppendorf), the supernatant poured off and the pellet washed with 70% ethanol. Spun again, ethanol poured off and the samples left to air dry overnight on the bench top.
10) Samples were resuspended in an appropriate amount of TE (pH 8).
2.3.1.2 Extraction of RNA

1) 0.5 g of fresh rosette leaves (4-6 week old plants), or seedlings from agar plates, was ground well in liquid nitrogen and transferred to a 2 ml microcentrifuge tube.

2) 1 ml of Trizol (Gibco BRL) was added, the sample vortexed and incubated at room temperature for 5 min.

3) 0.2 ml of chloroform was added and the tubes shaken vigorously for 15 sec. Samples were incubated at room temperature for 3 min.

4) Samples were spun at 4°C for 15 min in a microcentrifuge, and the aqueous layer transferred to a new tube.

5) RNA was precipitated by the addition of 0.5 ml isopropanol and incubation at 4°C for 10 min.

6) The samples were centrifuged at 4°C for 10 min in a microcentrifuge to pellet the RNA.

7) The RNA pellet was washed once with 70 % ethanol followed by centrifugation at 4 °C for 5 min.

8) The pellet was air dried briefly and resuspended in an appropriate amount of sterile water.

RNA was used directly for northern blotting or DNase treated for RT-PCR.

2.3.1.2.1 DNase treatment of RNA

RNA (16 μl of 5 μg/μl) was transferred to a microcentrifuge tube and the following added: 2 μl MgCl₂, 1 μl 1 M Tris-HCl pH 8.0, 1 μl DNase I, 0.5 μl RNasin. The reaction was allowed to proceed for 10 min at 37 °C. 0.5 μl 0.5 M EDTA was added. This sample was then used for RT-PCR.

2.3.1.3 mRNA isolation

mRNA was purified from total RNA using the Clontech NucleoTrap® Nucleic Acid Purification Kit (mRNA NucleoSpin mini kit). A total RNA solution obtained using the Trizol method above was used as the starting material, and the manufacturers' protocol was followed. All centrifugation was carried out in an Eppendorf microcentrifuge.
1) The same volume of 2x RM1 buffer was added to process 200-500 μl total RNA solution.
2) The NucleoTrap mRNA Suspension was resuspended by vortexing, and 15 μl of suspension added per 100 μg total RNA.
3) The tube was vortexed, heated to 68°C for 5 min, and then incubated at room temperature for 10 min, the tube was inverted every 2 min.
4) Centrifuged for 15 sec at 5000 rpm, then at maximum speed for 5 min. The supernatant was discarded.
5) The pellet was dissolved in 600 μl buffer RM2.
6) The suspension was transferred to a NucleoSpin Microfilter and microcentrifuged for 15 sec at 5000 rpm, then maximum speed for 2 min. Flowthrough was discarded.
7) 500 μl buffer RM3 was added to the microfilter and the beads resuspended by pipetting up and down.
8) Centrifuged for 2 min maximum speed. Flowthrough was discarded.
9) Steps 7-8 were repeated.
10) The tube was spun for an additional minute to remove the wash buffer.
11) The spin column was transferred to a new 1.5 ml tube. 20 μl pre-warmed RNase-free water was added per 10 μl beads. The beads were resuspended. The tube was incubated at 68°C for 10 min.
12) Centrifuged for 1 min and the eluate collected. Purified mRNA was stored at -80°C

2.3.2 Analysis of nucleic acids

2.3.2.1 Agarose gels

All PCR products and DNA samples were run on TAE 0.8% Gibco BRL electrophoresis grade agarose gels. Stocks of 50x TAE were made and diluted as appropriate. For 50x stock 242 g Tris base was added to 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA pH 8.0, this was adjusted to pH 7.2 and made up to 1 litre. A 1x solution was made from this and used for the gel and buffer. TAE and agarose were measured into a flask and heated in a microwave to melt the agarose, the gel was poured once cool. DNA samples were run alongside a Gibco 1 kb ladder.
Three DNA staining methods were used.

Ethidium bromide: 2 µl Ethidium bromide (EtBr) (10 mg/ml) was added directly to the gel tray then mixed with molten agarose. The gel was visualised under UV.

SYBR® Green I (Molecular Probes): this method allows bands to be detected under blue light with an orange filter, thus preventing UV damage of the DNA, which is essential when potentially excising the band for cloning and complementation; the agarose gel was poured and allowed to set. Stock SYBR® Green I was diluted 1:100 in 1x TAE (stored 4 °C up to 4 weeks), a 2:1 loading dye:diluted SYBR® Green I was made and left for a few minutes before mixing with the sample to be loaded.

Crystal Violet (Invitrogen): this method was employed for the running of PCR products to be cloned specifically by TOPO cloning (section 2.3.9). Agarose (0.4 g) was added to 50 ml 1x TAE and heated until dissolved. Crystal violet solution (30 µl of 2 mg/ml) was added, mixed and the gel poured. 8 µl 6x crystal violet loading buffer was added to 40 µl PCR product and loaded onto the gel. Bands appear as a blue colour.

2.3.2.2 Quantification of nucleic acids

DNA was quantified by running on an EtBr gel next to a X Hind IW/Eco RI ladder. The quantity of DNA in each band of the ladder is known, the sample is compared directly to the ladder and the band of equivalent intensity is used to determine the quantity of DNA.

RNA was quantified spectrophotometrically in solution. Absorbance readings were taken and subjected to the following equation: $A_{260} \times 40 \times \text{dilution factor (e.g. 500 in diluted 1:500)} = \text{RNA \(\mu\text{g/ml}\)}$

2.3.2.3 Product extraction from agarose gels

Three different methods were used for the extraction of a product from an agarose gel. The Qiagen geneclean kit uses a spin column method, whereas BIO101 uses glassmilk to extract the DNA. The Invitrogen genecleaning method again uses spin columns, this method was specifically used for extraction of fragments from crystal
violet gels for TOPO cloning (section 2.3.9). All geneclean kits were used according to manufacturers’ instructions.

2.3.3 Restriction digests
Digestion of DNA with restriction enzymes provides known end sequences of the DNA for ligation to other fragments. It is also useful to identify the presence of known sequence, a predicted series of bands will be observed on a gel; this is particularly useful when excising insert fragments from vectors.

A standard restriction digest was carried out as follows:
1 μl 10x restriction digest buffer (appropriate to enzyme to be used), 0.5 μl restriction enzyme, 3.5 μl water and 5 μl plasmid prep DNA were combined in a microcentrifuge tube and incubated overnight at the temperature appropriate for the enzyme being used (generally 37°C). Shorter incubations can be used, 1 unit of enzyme digests 1 μg DNA every hour.

2.3.4 PCR reactions
The conditions of PCR vary with the sample to be amplified and the primers used for amplification. However, standard volumes of reaction mix contents (to a total of 20 μl) were used for most PCRs:

2.3.4.1 Red Hot Taq (AB)
2 μl 10x PCR buffer
1 μl MgCl₂
2.5 μl dNTPs (2 mM each)
1 μl primer 1 (10 μM)
1 μl primer 2 (10 μM)
1 μl DNA
0.2 μl Red Hot Taq
11.3 μl water
### 2.3.4.2 Expand™ High Fidelity PCR System

2 µl 10x Expand HF buffer (with 15 mM MgCl₂)

0.4 µl dNTPs (10 mM each)

0.4 µl primer 1 (10µM)

0.4 µl primer 2 (10µM)

0.5 µl Expand Mix

1 µl DNA

15.3 µl water

The above were combined in a 0.5 ml microcentrifuge tube, mixed and overlaid with mineral oil. PCR was carried out in a Perkin-Elmer Thermocycler.

### 2.3.5 RT-PCR (Reverse Transcription-PCR)

RT-PCR was used to amplify products from RNA, therefore amplifying only those regions which are expressed in the plant.

1) 2 µl total RNA (0.5 µg/µl) was incubated with 1 µl 3’ primer and 10 µl water at 70°C for 10 min and placed immediately on ice.

2) To this sample the following were added; 4 µl 5x 1st strand synthesis buffer, 1 µl dNTPs (25 mM), 2 µl DTT, 1 µl superscript II (Gibco BRL) (200 U/µl).

3) The reaction was incubated at room temperature for 10 min followed by 42°C for 50 min.

4) 1 µl RNaseH was added and incubated at 37°C for 30 min.

5) All of the reaction (20 µl) was used in a PCR reaction of total volume 50 µl.

PCR reaction; 20 µl sample; 3 µl buffer 2 (Boehringer Expand™ High Fidelity PCR system); 1 µl dNTPs (25 mM), 1 µl each primer; 0.25 µl Taq Expand (Boehringer Expand™ High Fidelity PCR system); 23.75 µl water. A PCR using DNA was set up simultaneously as a positive control for the PCR.
2.3.6 Genome walking

Universal GenomeWalker™ Kit (Clontech).

DNA was extracted using the simplified Doyle and Doyle method described above (section 2.3.1.1.2), the concentration was determined spectrophotometrically and the sample diluted to a working concentration of 1 μg/μl. The quality of the DNA was checked according to the GenomeWalker protocol, by running 1 μl next to the provided control DNA on an EtBr agarose gel, checking that the resulting band was greater than 50 kb in size.

The purity of the DNA was assessed by a restriction digest using Dra I. The following were combined in a 0.5 ml tube: 5 μl genomic DNA, 1.6 μl Dra I (10 Units/μl), 2 μl 10x restriction buffer for Dra I, 11.4 μl water. The contents of the tube were mixed gently by inverting the tube, and the sample was incubated at 37°C overnight. 5 μl was run on an EtBr gel to confirm digestion of the DNA.

The procedure for genome walking was followed as in the manufacturers’ protocol. Blunt ended genomic DNA fragments were created by restriction digests in separate tubes by 5 enzymes; EcoK V; Sca I; Dra I; Stu I; Pvu II. 5 μl was checked on an EtBr agarose gel. The DNA was purified according to the protocol (see below) and resuspended in 20 μl TE (10/0.1, pH 7.5).

Purification of DNA

To each reaction tube 95 μl phenol was added. The tubes were vortexed at slow speed for 5-10 sec, and spun briefly to separate the layers. The top aqueous layer was transferred to a fresh tube with the addition of 95 μl chloroform. Again the tubes were vortexed, spun and the aqueous layer transferred to a new tube. To each tube the following were added: 190 μl ice cold 95% EtOH, 9.5 μl 3 M NaOAc (pH 4.5), and 20 μg glycogen. The tubes were vortexed briefly at slow speed and then spun at 15,000 rpm for 5 min. The pellet was washed in 100 μl ice cold 80% EtOH, spun at 15,000 rpm for 5 min and air dried. The pellet was dissolved in 20 μl TE (10/0.1, pH 7.5).
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The GenomeWalker Adapter was ligated to 4 µl of each purified DNA sample. In a 0.5 ml tube 1.9 µl GenomeWalker adapter (25 µM), 1.6 µl 5x ligation buffer and 0.5 µl T4 DNA ligase (1 unit/µl) were added to 4 µl purified DNA. The tubes were incubated overnight at 16°C. The reactions were stopped by placing the tubes at 70°C for 5 min. 72 µl TE (10/1, pH 7.4) was added to each tube.

PCR reactions were set up according to the manufacturers protocol using Advantage Taq Polymerase mix as recommended:

Primary PCR, 10 reactions:
- 378 µl deionized water
- 50 µl 10x Tth PCR reaction buffer
- 10 µl dNTP (10 mM each)
- 22 µl Mg(OAc)_2 (25 mM)
- 10 µl AP1 (10 µM)
- 10 µl Advantage Tth Polymerase mix (50x)

The secondary PCR mastermix was made as above with AP2 replacing AP1.

The tubes were mixed and 48 µl dispensed into labelled tubes. 1 µl of the appropriate gene specific primer (e.g. BAR1 or BAR2 see appendix1) was added to each tube along with 1 µl DNA. Tubes were overlaid with mineral oil and placed in a Perkin-Elmer thermocycler.

Thermocycler cycles:

Primary (1°) PCR;
- 94°C 25 sec; 72°C 3 min; 7 cycles
- 94°C 25 sec; 67°C 3 min; 32 cycles
- 67°C 7 min; 1 cycle

Secondary (2°) PCR, using 1 µl of the primary PCR diluted 1 in 50;
- 94°C 25 sec; 72°C 3 min; 5 cycles
- 94°C 25 sec; 67°C 3 min; 20 cycles
- 67°C 7 min; 1 cycle.
Using positive controls (human DNA which had also been ligated at the same time as the DNA samples of interest, and a preconstructed library supplied with the kit) a series of alterations was made to the published procedure to successfully PCR amplify the positive control. Adjustments were made to the PCR cycles, reducing the annealing temperatures, and the time for which these temperatures were held. Two rounds of PCR were performed, 1 µl of undiluted primary PCR product was used in the secondary PCR (manufacturers' protocol recommended dilution of 1:50). The successful PCR cycles to amplify the positive controls were as follow:

1° PCR:  
- 94°C, 17 sec; 70°C, 3 min; 7 cycles
- 94°C, 17 sec; 65°C, 3 min; 37 cycles
- 65°C, 7 min; 1 cycle

2° PCR:  
- 94°C, 17 sec; 70°C, 3 min; 5 cycles
- 94°C, 17 sec; 70°C, 3 min; 24 cycles
- 65°C, 7 min; 1 cycle.

The genomic DNA restriction libraries were subjected to the conditions above, using the GUS primer for one end of the T-DNA (primer GUS5′1 -figure 3.9; appendix 1). No bands were obtained for the genomic libraries. In an attempt to further improve the genome walking efficiency 5% DMSO was added to the 1° reaction mix, without success. However, when the DMSO modified PCR was performed using a primer to the end of the T-DNA containing the gene for basta resistance, a very non-specific banding pattern was obtained. Genome walking was repeated using the same conditions with the exclusion of DMSO. It was this method that produced the product which successfully identified the GIL1 gene. The bands were excised from this gel extracted from the agarose using the Qiagen geneclean kit (section 2.3.2.3). The purified product and the appropriate primers (AP2 (adapter primer) and BAR2 (figure 3.9); appendix 1) were sent to the automated sequencing service of Leicester University. Sequencing was achieved using fluorescent labelling.
2.3.7 5' and 3' RACE (Rapid Amplification of cDNA Ends)

The SMART™ RACE cDNA Amplification kit method from Clontech was used for amplification of both 3' and 5' cDNA ends. The manufacturers protocol (available from the web site www.clontech.co.uk) was followed. The SMART II oligonucleotide, 3'-RACE cDNA synthesis primer (3'-CDS), 5'-RACE cDNA synthesis primer (5'-CDS), Universal primer mix (UPM), nested universal primer (NUP) and gene specific primers (GSP1 (a 3' primer for the 5'RACE reaction) and GSP2 (a 5' primer for the 3'RACE reaction)) were synthesised, as in the manufacturers' protocol, by Bioline (see appendix 1 for primer sequences).

Total RNA used for this method was isolated using the Trizol method, mRNA was purified using the Clontech NucleoTrap® Nucleic Acid Purification Kit.

First strand synthesis:
The following were combined in separate 0.5 ml centrifuge tubes:
5'RACE; 1-3 μl total or poly A+ RNA (1 μg); 1 μl 5'-CDS primer; 1 μl SMART II oligo.
3' RACE; 1-3 μl total or poly A+ RNA (1 μg); 1 μl 3'-CDS primer.
Sterile water was added to a final volume of 5 μl.
The contents were mixed and the tubes spun briefly to collect the contents before incubation at 70°C for 2 min.
The tubes were cooled on ice for 2 min, and spun briefly.

The following were added to each tube:
2 μl 5x First-Strand buffer (Gibco BRL)
1 μl DDT(20 mM)
1 μl dNTP Mix (10 mM)
1 μl Superscript II (Gibco BRL)

Contents were mixed by pipetting, tubes spun and incubated at 42°C for 1.5h.
TE pH 8.0 (100 μl) was added to samples containing total RNA. mRNA samples were divided into two tubes each, to one set of tubes 125 μl TE pH 8.0 was added.
(as recommended in the manufacturers' protocol) and to the other set 50 μl TE pH 8.0 was added. Tubes were then incubated at 72°C for 7 min. Samples were stored at -20°C.

The following PCR reactions were set up;

<table>
<thead>
<tr>
<th>5’RACE</th>
<th>3’RACE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μl synthesised first strand</td>
<td>1 μl synthesised first strand</td>
</tr>
<tr>
<td>0.4 μl NUP</td>
<td>0.4 μl NUP</td>
</tr>
<tr>
<td>0.4 μl GSP1</td>
<td>0.4 μl GSP2</td>
</tr>
<tr>
<td>0.4 μl dNTPs (10 mM each)</td>
<td>0.4 μl dNTPs (10 mM each)</td>
</tr>
<tr>
<td>2 μl PCR Buffer 2 (Expand PCR system)</td>
<td>2 μl PCR Buffer 2</td>
</tr>
<tr>
<td>15.3 μl water</td>
<td>15.3 μl water</td>
</tr>
<tr>
<td>0.5 μl Expand High fidelity PCR system</td>
<td>0.5 μl Expand High Fidelity PCR system</td>
</tr>
</tbody>
</table>

The tubes were overlaid with mineral oil. The Taq was added once the tubes had reached 94°C as a hot start.

The following PCR cycles were used:

94°C 30 sec; 72°C 3 min; 5 cycles
94°C 30 sec; 72°C 30 sec; 72°C 3 min; 5 cycles
94°C 30 sec; 68°C 30 sec; 72°C 3 min; 25 cycles.

Amplification of the 5’ RACE product was not possible with the RNA or mRNA samples originally created. A new first strand synthesis was performed with slightly different parameters than those suggested in the manufacturers' protocol.

The new procedure was performed as follows:
Into a microcentrifuge tube:

5 μg Col RNA
5 μl CDS-5’
Chapter 2 Materials and Methods

5 µl SMART II oligo
1.5 µl water
70°C 2 min, ice 2 min.

The following was added to each tube:
4 µl 5x synthesis buffer
2 µl DTT (100 mM)
1 µl dNTPs (10 mM each)
42°C 60 min, 70°C 15 min.
This sample was stored at -20°C until required.

1 µl of the first strand synthesis product was used in a 20 µl PCR.

2.3.8 Northern blotting

RNA samples were run on formaldehyde gels.
For a 150 ml gel tray, 130 ml water, 15 ml 10x MOPS (0.2 M MOPS (Sigma), 0.05 M NaAc (pH 7.0), 0.01 M EDTA (pH 8.0) adjusted to pH 7.0), and 1.8 g electrophoresis grade agarose were heated in a microwave until all of the agarose had melted. After cooling to 50°C, 7.65 ml 40% formaldehyde was added, mixed and the gel poured in a fume cupboard.

RNA loading dye was prepared in advance- 2.5 µl 10x MOPS, 12 µl 100% formamide, 4 µl 40% formaldehyde and 0.5 µl tracking dye. Aliquots were stored at -20°C. Before use loading dye was mixed 2:1 with EtBr.

RNA samples were heated to 65°C for 10 min and cooled on ice before mixing with 3 µl loading buffer/EtBr.

Gels for northern blotting were run, in a fume cupboard, at 60V (3-4V per cm gel) using 1x MOPS as running buffer. Visualisation was achieved using a UV transilluminator.
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Gels were transferred into 400 ml wash solution (20 ml 20x SSC (175.3 g NaCl and 88.2 g sodium citrate dissolved in 800 ml water, adjusted to pH 7.0 and made up to 1 l), 0.8 g NaOH) briefly, and then blotted overnight using 10x SSC.

Membranes were air dried and UV crosslinked in a UV Stratalinker 2400.

2.3.8.1 Radiolabelling and detection

2.3.8.1.1 Oligolabelling a probe

Probes were labelled using the Pharmacia oligolabelling kit.

DNA (25-50 ng in no more than 34 µl TE) was denatured at 100°C for 2 min and cooled on ice.

The following were added: 10 µl reagent mix, 5 µl [α-32P] dCTP (1.87 MBq), and water to a total volume 49 µl. 1 µl Klenow was added and mixed gently by hand. This was incubated at 37°C for 60 min.

The DNA was denatured at 100°C for 2 min and cooled on ice before addition to the prehybridisation solution.

2.3.8.1.2 Probing a blot

Blots were prehybridised for 2h in Church buffer (0.5 M Na2HPO4 pH 7.4, 1 mM EDTA, 7% SDS). The probe was added after 2h for hybridisation overnight. The following day the blots were washed with 2x SSC, 0.1% SDS, washing was repeated 2-3 times until no more radioactivity was detected in the wash solution.

Membranes were wrapped in surranwrap and placed in an autoradiography cassette with X-ray film. The film was developed in an automatic developer.

2.3.8.2 Alternative northern blot labelling and detection

The UV cross-linked membrane was prehybridised in 5 ml preheated (65°C) Clontech ExpressHyb Hybridization Solution in a screw top Hybaid tube for 2h at 65°C in a Hybaid oven. The probe was labelled as described above. The prehybridisation solution was replaced after 2h, the probe added and incubated for
2h at 65°C. The oven temperature was reset to 55°C and the door left open while the membrane was washed 3 times for 15 min each in 2x SSC, 0.2% SDS. The door to the oven was then shut while the membrane was washed twice with 0.1x SSC, 0.1% SDS for 20 min each. The membrane was checked with the Geiger counter between the two final washes to check levels of radiation.

The membrane was then sealed in polythene and placed in a phosphoimaging cassette; this was scanned after an overnight exposure, and if necessary the exposure repeated to obtain an optimum level.

### 2.3.9 Cloning

#### 2.3.9.1 Vectors

Three vectors were used for cloning; bluescript (pBS), pcr®-XL-TOPO® (Invitrogen) and a modified pGEM vector renamed pDK101. Bluescript and pGEM contain the lacZ gene which allows blue/white selection of recombinants. The TOPO cloning vector carries a gene which is lethal unless insertion of DNA has occurred, so colonies should contain the plasmid with insert.

pGEM®-72f(+/-) (Promega) had been modified to create pDK101 by the removal of the restriction cut site and insertion of an Xcm I cut site; cutting with this enzyme leaves a single T base overhang for A-T cloning of PCR products.

#### 2.3.9.2 Vector dephosphorylation

To prevent re-ligation of the digested vector the ends were dephosphorylated. 1 μg DNA was digested in a 30 μl reaction. 1 μl of a 1 in 5 dilution of phosphatase in TE buffer (5U) was added and incubated for 20 min at 37°C. 0.2 μl 0.5 M EDTA (pH 8.0) was added and heated for 10 min at 65°C. The volume was made up to 50 μl and phenol:chloroform extracted. The DNA was ethanol precipitated and washed with 70% EtOH. The pellet was dried and resuspended in 20 μl TE.
2.3.9.3 Ligation (using pGEM and pBS)
For a 15 μl ligation, 10 μl DNA (50 ng), 1 μl T4 ligase (Gibco BRL), 3 μl 5x buffer and 1 μl vector were mixed in a 0.2 ml microcentrifuge tube. Ligation was allowed to proceed at 4°C overnight.
The ligated DNA was precipitated the following morning with 1.5 μl tRNA, 50 μl EtOH, and resuspended in 50 μl water.

2.3.9.4 Ligation (using TOPO vector)
4 μl of gene cleaned PCR product was combined with 1 μl pcr®-XL-TOPO® vector in a microcentrifuge tube. The tube was mixed gently and incubated for 5 min at room temperature. 1 μl 6x TOPO® cloning stop solution was added and mixed. The tube was briefly centrifuged and placed on ice ready for transformation.

2.3.9.5 Transformation of electrocompetent cells
3 μl of resuspended ligation was added to 40 μl of competent cells on ice. This was transferred to a disposable electroporation cuvette (Eppendorf Scientific Inc.). Electroporation used the BIORAD Gene Pulser® Electroporation System using high range resistance with the capacitance set at 25 μF. LB (1 ml) was added immediately and transferred to a sterile universal bottle. The cells were allowed to recover for 1h in a 37°C shaking incubator.

Dilutions (1/10 and 1/100), and the concentrated transformation were plated onto LB plates containing IPTG, X-GAL and ampicillin (section 2.2.2.4). Plates were incubated at 37°C overnight. White colonies were selected.

2.3.9.6 Transformation of electrocompetent TOPO10 cells
This method was used specifically for transformation of TOPO10 cells by the TOPO® vector (Invitrogen).
Ligation solution (2 μl) was added to 80 μl of competent cells on ice. This was transferred to a 0.2 cm disposable electroporation cuvette. Electroporation used the
BIORAD Gene Pulser® Electroporation System using low range resistance of 400Ω with the capacitance set at 25 μF and the preset voltage 2.5 kV. SOC medium (1 ml) was added immediately and the cells incubated at 37°C in a shaking incubator for 1 hour. 40 μl of cells were plated onto LB containing kanamycin (50 μg/ml). The remaining cells were spun down, the supernatant poured off, and the cells resuspended in the remaining culture medium for plating onto a second plate. Plates were incubated overnight at 37°C. Colonies which grow should be recombinants; 10 were selected from each plate for confirmation of the presence of the insert within the vector.

2.3.9.7 Transformation of calcium competent cells

1 μl ligation was added to 200 μl calcium competent cells in a microcentrifuge tube and stored on ice for 15 min. The cells were heat shocked by incubation at 42°C for 90 sec, tubes were returned to ice. LB (1 ml) was added and the cells incubated at 37°C for 1 hour to recover. A 1:10 dilution was plated, the remaining contents were centrifuged briefly and the supernatant poured off. The cells were resuspended in the small amount of supernatant remaining and plated onto LB with the appropriate antibiotic, IPTG and X-GAL. White colonies were selected.

2.3.9.8 Identification of recombinants

Isolation of recombinants was confirmed for PBS either by an Eco RI restriction digest of a plasmid preparation (section 2.3.10), or PCR using T3 and T7 primers. Colonies were selected and resuspended in 50 μl water, the tube was boiled for 10 min, and then transferred to ice. A stab plate was made of the colonies for future use. DNA (1 μl) was pipetted into a tube with the following: 1 μl buffer, 0.5 μl MgCl₂, 1.25 μl dNTP (2 mM), 0.5 μl each primer, 0.1 μl Red Hot Taq (Advanced Biotechnologies) 5.15 μl water. Thirty PCR cycles were performed as follow: 95°C 1 min, 55°C 1 min, 70°C 3 min. The presence of the insert was confirmed by a PCR band, of a size matching that of the inserted fragment, on an agarose gel.
Confirmation of recombinants for pGEM was achieved by PCR (as above) using the same primers as used to isolate the fragment for insertion.

Recombination was confirmed for TOPO cloning again by PCR. Colonies were resuspended in 10 µl Expand PCR mix (1 µl buffer 2, 0.2 µl dNTPs (10 mM), 0.2 µl each primer, M13 forward and reverse (10 mM), 0.25 µl Expand Taq, 8.15 µl water). The pipette tip used for picking the colony was stabbed onto a LB + kanamycin plate which was incubated at 37°C overnight. The resuspended colony in PCR mixture was vortexed and spun briefly, 2 drops of mineral oil were added and the following cycles utilised on a Perkin Elmer PCR machine; 94°C 10 min, 1 cycle; 94°C 45 sec, 55°C 45 sec, 72°C 1 min 30 sec, 25 cycles.

2.3.10 Plasmid preparation

This method was used to obtain plasmid for transformation and also to obtain recombinant plasmids for further analysis.

A fresh overnight culture was centrifuged at 4°C to pellet the cells. The supernatant was removed and the pellet volume estimated. The pellet was resuspended in 1 volume cold TEG (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0)) by vortexing. To this 2 volumes of lysis buffer (0.2% NaOH, 1% SDS), were added and the tube inverted until the solution cleared. 1.5 volumes cold 3 M KAc were added and stored on ice for 5 min. The sample was centrifuged for 5 min and the supernatant transferred to a new tube. Three volumes of EtOH were added to precipitate the plasmid. The plasmid was pelleted by centrifugation, the supernatant removed and the pellet allowed to dry for a short while. The pellet was resuspended in water and RNase was added.

2.3.11 Preparation of BAC clone for restriction digest

This method was recommended by the Ohio Stock Center for the clone K19M22 (section 3.4), which was supplied as a stab culture.

K19M22 was streaked onto LA containing kanamycin and grown overnight at 37°C. A 100 ml overnight culture was made containing 100 µl kanamycin 10 mg/ml. The culture was centrifuged and the supernatant poured off. The pellet was resuspended
in the remaining culture medium, centrifuged again and the supernatant removed. The pellet was resuspended in 0.2 ml 50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0, 5 mg/ml lysozyme. The sample was incubated on ice for 5 min. To this 0.4 ml 0.2 N NaOH, 1% SDS was added and incubated on ice for 5 min. 0.3 ml 3M KAc was added, mixed gently and incubated at -80°C for 15 min. The sample was centrifuged for 15 min. Supernatant (0.75 ml) was transferred to a new tube containing 0.45 ml isopropanol and incubated -80°C for 15 min. The DNA was pelleted by centrifugation for 5 min. The supernatant was removed and the pellet washed with cold 70% EtOH. The pellet was dried on the bench for 15 min, followed by the addition of 40 μl TE pH 8.0. The DNA was allowed to dissolve over night at 4°C.

2.3.12 Storage of positive recombinants / plasmids

10 ml LB containing the appropriate antibiotic was inoculated with a colony. The culture was grown overnight at 37°C in a shaking incubator and 0.5 ml was transferred to a microcentrifuge tube containing 0.5 ml 50% glycerol, 50% LB. The tubes were placed into liquid nitrogen to snap freeze the contents and transferred to -80°C for storage.

2.3.13 Preparation of competent cells

2.3.13.1 Electrocompetent cells

1 litre of LB was inoculated with 1/100 volume of a fresh overnight culture. Cells were grown at 37°C in a shaking incubator until the optical density was A₆₀₀ of 0.5-0.8. The culture was chilled on ice for 30 min, aliquoted into centrifuge tubes, and then centrifuged at 4°C for 15 min, 4000 x g. The supernatant was removed and the pellets resuspended in a total of 1 litre ice cold water. Centrifugation was repeated. Cells were resuspended in a total of 0.5 l ice cold water and the centrifugation repeated. Cells were resuspended in 20 ml ice cold water, 10% glycerol, and centrifugation was repeated. Finally the cells were resuspended in a volume of 2-3 ml ice cold 10% glycerol. Aliquots of cells were stored at -80°C.
2.3.13.2 Calcium competent cells

LB (200 ml) was inoculated with 2 ml from a fresh overnight 500 ml culture. The culture was grown at 37°C in a shaking incubator as above. The culture was cooled on ice for 30 min, aliquoted into cold centrifuge tubes, and centrifuged at 3700 rpm, 4°C for 5 min. The supernatant was discarded. Ice cold sterile 0.1 M CaCl₂ was added to 50% original volume and the tubes were stored on ice for at least 1h (overnight incubation is possible). The cultures were centrifuged at 3700 rpm, 4°C for 5 min, and resuspended in 20% volume 0.1 M CaCl₂. The cells were used immediately.

2.3.14 cDNA library screening

A Columbia rosette leaf library (Y1090r-) was obtained from Clontech. E.coli which contained the library was plated from glycerol stock onto LB plates containing 50 µg/ml Ampicillin and incubated at 37°C overnight.

From this plate one colony was selected and grown overnight at 37°C in 10 ml LB containing 10 mM MgSO₄ and 0.2% maltose.

The following day 50 µl diluted phage library was added to the overnight culture and incubated for 15 min at 37°C. 9 ml of this culture were added to 145 ml of top agar (55°C) and approximately 10 ml was spread onto each prewarmed (37°C) LB plate, allowed to dry and incubated at 37°C overnight.

Circular, 9 cm diameter, nylon membrane was laid onto each plate, a needle was used to stab through the membrane and agarose in 3 positions to allow easy realignment of the filters. The membrane was removed from the plate and washed in denaturing solution (1.5 M NaCl, 0.5 N NaOH), followed by neutralising solution (1 M Tris (pH 7.4), 1.5M NaCl), then 2x SSC. The membrane was placed onto 3MM Whatman paper to dry, and then UV crosslinked.
The filters were then probed and washed in the same way as a northern blot (section 2.3.8). The filters were taped to Whatmann 3MM paper, covered with surranwrap, and placed in an autoradiograph cassette with X-ray film.

Upon identification of positive clones on the autoradiograph in the first round screening, the film, filters and plates were all aligned and the area of the plate which contained the positive clone was selected with a wide bore pipette tip and transferred to a 1.5 ml microcentrifuge tube. 1 ml of 1x λ dilution buffer (diluted from a 10x stock to 10 mM Tris-HCl (pH 7.5), 10 mM MgSO₄) and 2 drops of chloroform were added and the bacteriophage particles allowed to elute at room temperature. A dilution series (1:10, 1:100, 1:1000, 1:10000) of the resuspended bacteriophage particles was made using 1x λ dilution buffer, 200 µl of Y1090r- cells added, mixed and incubated at 37°C for 30 min. These were plated onto prewarmed LB plates using top agar as above. The transfer to filters and probing was repeated and areas of the plates containing positive clones selected, until a stage was reached where the positive clone could be seen as an individual colony on the plate and isolated. The individual colony was selected, eluted and plated as before; this time the plate contained top agar instead of LB.

After incubation at 37°C overnight 3 ml of 1x λ dilution buffer were added to the plate to elute the bacteriophage particles at room temperature on a shaking platform for 2 hours. The eluate was poured into a centrifuge tube and spun at 2500 rpm for 10 min at 4°C. The supernatant was transferred to a new tube with the addition of 1 µl DNasel (1 mg/ml) and 1 µl RNase A (1 mg/ml) and incubated at 37°C for 15 min. An equal volume of a solution containing 20% PEG 8000 (polyethylene glycol) and 2 M NaCl in 1x λ dilution buffer was added and incubated on ice for 1h.

The tube was centrifuged for 10 min at 10000 x g 4°C, the supernatant was removed with a pipette and the tube inverted on tissue paper to remove the remaining liquid. The pellet was dissolved in 0.5 ml TE (pH 8.0) by vortexing, 5 µl 10% SDS was added and incubated for 5 min at 68°C. 10 µl 5 M NaCl was added and DNA
purified by extraction once with phenol:chloroform, and once with chloroform. The aqueous layer was transferred to a new tube; an equal volume of isopropanol was added, mixed, and stored at -70°C for 15 min. DNA was recovered by centrifugation for 15 min at 12000 x g 4°C. The pellet was washed with 70% ethanol, and air dried for a few minutes. The DNA was dissolved in 50 μl TE (pH 8.0).

The phage prep was restricted with the appropriate restriction enzyme (one which cuts out the complete fragment but which does not cut within the fragment, if the predicted sequence is known). For restriction digests the following were combined, 20 μl phage prep, 5 μl reaction buffer, 2 μl restriction enzyme, 23 μl water and incubated overnight at the temperature optimal for the restriction enzyme used. Half of the digest was run on an EtBr gel to determine the size of the fragment, the other half was run on a SYBR Green I gel so no exposure to UV light was required for excision of the fragment from the gel. Fragments isolated were cloned into an appropriate vector and sequenced.
3.0 *gilI* - A Mutant with Altered Gravitropic Responses to Light.

3.1 Introduction

The morphology of plants is determined by a number of environmental cues, for example, light and temperature. These environmental stimuli are unlikely to act independently to elicit changes in plant growth and it is therefore plausible that interaction/crosstalk occurs between their signalling pathways.

The establishment of the role of phytochrome A in Arabidopsis for regulating inhibition of hypocotyl elongation has been assisted by the identification and study of mutants such as *phyA*, *fhy1* and *fhy3*. *fhy1* and *fhy3* define components of the phytochrome A signalling pathway and the FHY1 and FHY3 proteins both play a role in R, requiring phyB (chapter 4). These mutants specifically define components of the VLFR or HIR respectively. Other mutants which affect particular branches of the phyA pathway would enhance the understanding of this pathway and perhaps its interaction with others.

One signal that is perceived by plants, and stimulates directional growth, is gravity. Emerging hypocotyls are negatively gravitropic, that is they grow at an angle opposed to the gravitational vector, this is true for dark grown hypocotyls and is therefore not a light-dependent response. Primary roots are usually positively gravitropic, they grow towards the gravitational vector. The organs of a mature plant are positioned at a particular angle relative to gravity, the gravitational set point angle (GSA) (Firn and Digby, 1997).

Hypocotyls of *Arabidopsis thaliana* are negatively gravitropic. However, hypocotyls grown under monochromatic R or FR are not negatively gravitropic, they grow in any orientation, this is referred to as randomisation. R light induced randomisation of Arabidopsis hypocotyls has been shown to be mediated by phyA and phyB, and no other phytochromes (Robson and Smith, 1996, Poppe et al, 1996). Under
continuous R phyB mutants and phyA mutants remain randomised, however the phyA phyB double mutant is negatively gravitropic. The randomisation controlled by phyB is R/FR reversible, whereas that controlled by phyA is a non-reversible VLFR (Poppe et al, 1996). The pathways utilised to mediate phytochrome induced changes in gravitropism remain elusive.

One approach to identify mutants which define points of interaction between light perception and gravity perception is to screen for those which display altered gravitropism. The isolation and characterisation of a mutant from a FR screen for gravitropic hypocotyls is presented in this chapter.

3.2 Results

3.2.1 Screening for a mutant with altered gravitropic responses to light

Hypocotyls of Arabidopsis display randomisation in response to R light; whether this response is transient or persists until perception of B or W is under investigation (Whitelam lab.). Hypocotyls are observed to randomise even under very low fluence rates (Poppe et al, 1996). A screen was developed by Dr. Praekelt (University of Leicester) that would effectively identify mutants that displayed a reduction in randomisation caused by very low fluence rates of FR. The successful procedure is described in section 2.1.7.1 and summarised in figure 2.1. Loss or reduction of the randomisation response could be mediated by phyA or phyB; however, under the conditions used, pulses of FR, phyA is likely to be the only photoreceptor. Existing FR insensitive mutants can be easily eliminated by using a secondary screen to isolate only those mutants which have a hypocotyl length similar to wt under continuous FR.

Versailles T-DNA tagged lines available from stock centres were screened as described in section 2.1.7.1. Candidate mutants were selected, grown in a greenhouse and the seed rescreened under the same conditions to confirm the phenotype. Dr. Praekelt isolated one mutant from the Versailles lines (figure 3.1a). This mutant consistently showed the "U-shaped" hypocotyl under screen conditions.
Chapter 3  
*gil1* - A Mutant with Altered Gravitropic Responses to Light

Figure 3.1a Hypocotyl orientation of wild type (WS) and *gil1*
following the plate rotation screen (FR pulses 5min/2h)

Under these conditions WT grows in a random orientation, whereas *gil1* displays a characteristic "U" shape, by responding to gravity as the plate is rotated.

For more details of plate rotation screen see section 2.1.7.1

Figure 3.1b Hypocotyl orientation of WS and *gil1*
following 3d continuous R

Under these conditions WT grows in a random orientation, whereas *gil1* responds to the gravity vector by growing upwards

(photographs courtesy of Dr. U. Prackelt)
The mutant selected was also grown on horizontal agar plates under continuous FR and continuous R (figure 3.1b), with comparison to wild type. The mutant was not insensitive to FR or R as shown by the fact that de-etiolation was not greatly impaired. However, it appeared that the mutant was less randomised under R (figure 3.1b). Hence the mutant was named \textit{gill} (\textit{gravitropic in light 1}).

\textit{gill} as the pollen donor was backcrossed to wt (WS) (Dr. Praekelt, pers. com.). The F1 seed was grown in the presence of kanamycin and all seedlings were resistant; this confirms that all of the F1 seeds are the result of true crosses. The F1 was selfed, and again grown in the presence of kanamycin, this F2 population displayed kanamycin resistance in a 3:1 ratio, consistent with a T-DNA insertion at a single locus. Over 300 F2 plants were selfed and seeds from individual plants were divided between two treatments. One plate contained kanamycin, seeds on this plate were grown under white light to confirm that all were resistant to kanamycin. The second plate did not contain kanamycin, the seeds on this plate were grown in R to look for the negatively gravitropic \textit{gill} phenotype. Populations which contained the \textit{gill} mutation were 100% kanamycin resistant, suggesting that the T-DNA insertion may cause the mutation (Dr. Praekelt, pers. com.).

3.2.2 \textit{gill} Mutant Phenotype

3.2.2.1 \textit{gill} phenotype under screen conditions

The \textit{gill} mutant grown under a continuous light source of FR or R on horizontal plates demonstrates less randomisation than wild type; however, \textit{gill} is not constitutively gravitropic as it displays a small amount of randomisation in some seedlings.

The phenotype of \textit{gill} is obvious with the rotation of the agar plate (figure 3.1a). However, it is not easy to quantify the reduction of randomisation in this way. To determine the magnitude of randomisation occurring in wild type, and how this was affected in \textit{gill}, it was necessary to develop a different approach to observe randomisation. The method used to determine the degree of randomisation is described in section 2.1.7.2.
Figure 3.2 Hypocotyl angle of seedlings following 3d FR pulses (5 min/30 min)

a) WS (wild type)  
b) gill

Photograph shows an example of one plate under these conditions, 60 seedlings were measured for each graph.

For details of angle measurements see section 2.1.7.2

Randomisation of gill is greater than that of wt under these conditions.
Figure 3.2 shows the effect of pulses of FR on the orientation of gill compared with wild type. The distribution of hypocotyl angles is greater in the wild type than in gill. Wt seedlings responded to pulses of FR by growing at angles between 0 and 170°. In contrast the majority of gill hypocotyls grow at an angle between 0 and 35°.

3.2.2.2 Phenotype of gill grown in the dark
Seedlings grown in the dark are negatively gravitropic. To confirm that this remained true for gill randomisation in the dark was assessed (figure 3.3a). A small degree of randomisation occurred in wt, this could be due to the germinating W light pulse. WS seedlings were screened again without a germinating W light pulse (figure 3.3b). Both WS and gill are negatively gravitropic in the dark, suggesting that the randomisation does occur in response to light. Both wt and gill respond strongly to the gravity vector in the dark.

3.2.2.3 Randomisation in continuous FR
The reduced randomisation of gill is evident under VLFR conditions (pulses of FR); this response may be more or less apparent under different light regimes; gill was screened under continuous FR. The procedure remained the same as for pulses of FR (above), following germination the plates were transferred to continuous FR for 3d. The results of this experiment are presented in figure 3.4. Again gill displays less randomisation than wt in terms of angle from the vertical; however, randomisation of gill is greater under continuous FR than under pulses of FR.

3.2.2.4 Randomisation in R (continuous and pulses)
The above procedure to investigate randomisation was applied to both continuous R and pulses (5 min every 2h) of R. The results are presented in figure 3.5a for continuous R and figure 3.5b for pulses of R. The lack of gill randomisation in R is more dramatic than in continuous FR. The hypocotyls of gill vary up to only 60° from the vertical under both Rc and Rpulses, whereas wt demonstrates randomisation up to 175° from the vertical.
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Figure 3.3a Hypocotyl orientation following dark  
a) WS (wild type) b) *gill*  
56 seedlings were measured for each graph

Figure 3.3b Hypocotyl orientation of WS (wild type) following dark,  
with no germinating W light pulse. 28 seedlings measured  
for details of angle measurements see section 2.1.7.2  

Some randomisation occurs in dark grown plants (a), this is caused by the  
white light pulse given for synchronous germination. In the absence of a  
white light pulse less randomisation occurs (b).
Figure 3.4 Hypocotyl angle of seedlings following 3d continuous FR

a) WS (wild type) b) gill

Photograph shows an example of one plate under these conditions, 87 seedlings were measured for each graph
for details of angle measurements see section 2.1.7.2

Randomisation of WS is slightly greater than that of gill under continuous FR. However, randomisation of gill is greater under these conditions than under other monochromatic light sources (figures 3.2-3.5).
Figure 3.4 Hypocotyl angle of seedlings following 3d continuous FR
a) WS (wild type) b) gill
Photograph shows an example of one plate under these conditions,
87 seedlings were measured for each graph
for details of angle measurements see section 2.1.7.2
Randomisation of WS is slightly greater than that of gill under continuous FR. However, randomisation of gill is greater under these conditions than under other monochromatic light sources (figures 3.2-3.5).
Figure 3.5a Hypocotyl angle of seedlings following 3d continuous R
a) WS (wild type) b) gill
Photograph shows an example of one plate under these conditions,
28 seedlings were measured for each graph
for details of angle measurements see section 2.1.7.2

gill is much less randomised than WS under continuous R.
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Figure 3.5b Hypocotyl angle of seedlings following 3d R pulses (5 min/2 h)

a) WS (wild type) b) *gil1*

Photograph shows an example of one plate under these conditions, 25 seedlings were measured for each graph

for details of angle measurements see section 2.1.7.2

WS displays greater randomisation than *gil1* under pulses of R.
A summary of the mean and standard deviation values for randomisation under the different conditions used is shown in table 3.1. For the purpose of this table any negative values (for hypocotyls which grew towards the left of the plate) were converted to positive values. This provides information concerning the angle of growth rather than the average of the directions of growth.

<table>
<thead>
<tr>
<th>light treatment</th>
<th>WS mean</th>
<th>SD</th>
<th>gill mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR pulses</td>
<td>33.8</td>
<td>38.7</td>
<td>11.3</td>
<td>9.5</td>
</tr>
<tr>
<td>FR continuous</td>
<td>51.2</td>
<td>28.6</td>
<td>34.2</td>
<td>28.0</td>
</tr>
<tr>
<td>R pulses</td>
<td>51.3</td>
<td>36.6</td>
<td>20.4</td>
<td>14.9</td>
</tr>
<tr>
<td>R continuous</td>
<td>87</td>
<td>49.6</td>
<td>19.3</td>
<td>14.1</td>
</tr>
</tbody>
</table>

Table 3.1 Randomisation of wt and gill under different light regimes.

3.2.2.5 Hypocotyl length of gill

Mutants with impaired phyA or phyB signalling pathways often demonstrate an altered de-etiolation response in the appropriate light conditions e.g. fhy1, fhy3 have a reduced de-etiolation response in FR. gill demonstrates a reduction in phyA mediated randomisation in FR, and a reduction in phyA or phyB mediated randomisation in R; it is therefore possible that other phyA (and phyB) responses are impaired in gill.

Seeds of gill and wt were germinated as described in section 2.2.1. After the final 24h dark period of germination plates were transferred, for 3d, to pulses of FR (dark controls wrapped in foil in FR source), or pulses of R. No obvious differences between hypocotyl lengths of wild type and gill were observed in dark or R pulses (figure 3.6). However, after 72 h under FR pulses gill hypocotyls were 1-2 mm shorter than those of wt, this phenotype was not observed after shorter time periods. So it is possible that the de-etiolation process under FR is enhanced in gill.
Figure 3.6 Average hypocotyl length of WS (wild type) and *gill* under a range of conditions
a) dark b) FR pulses (5 min/30 min)
c) R pulses (5 min/30 min) d) R pulses (5 min/2 h)
Error bars represent the value for 95% confidence interval
Under dark or R pulses there is no difference in hypocotyl length between *gill* and wt. However, under FR pulses *gill* is slightly longer than wt after 72h.
Figure 3.7 Average hypocotyl length of WS (wild type) and gill under different fluence rates of continuous FR. 30 seedlings were measured for each point. Error bars represent value for 95% confidence interval. gill does not display any difference in hypocotyl length compared to wt over a range of FR fluence rates.
The possibility of the enhancement of de-etiolation in *gill* seedlings being greater under different fluence rates of light was investigated. Seedlings were grown for 3d under different fluence rates of FR, ranging from 0.04 μ mol m$^{-2}$ s$^{-1}$ to 4.17 μ mol m$^{-2}$ s$^{-1}$ (figure 3.7). Under these conditions no difference in hypocotyl length was observed between WS and *gill* (figure 3.7). *gill* may therefore have some enhancement of de-etiolation under particular light regimes, such as FR pulses, but this characteristic is not constitutively displayed.

### 3.2.2.6 Cotyledon angle of *gill*

Hypocotyl length was not dramatically affected in *gill*. However, other de-etiolation responses, such as cotyledon opening, could be affected in *gill*. The angle between the cotyledons of wild type and *gill* was measured. Figure 3.8 shows that in *gill* this de-etiolation response is unaffected in FR pulses and in R pulses (5 min/2h). However, the opening of cotyledons in *gill* under more frequent pulses of R (5 min/30 min) does vary slightly from that of wt. At 24h *gill* cotyledons are less open than those of wt, but by 72h the cotyledons of *gill* are slightly more open than those of wt. This suggests that under frequent pulses of R the de-etiolation process in *gill* is initiated later than in wt, but by 48h these seedlings show normal de-etiolation. Enhancement of de-etiolation in *gill* by FR pulses is not manifested in the opening of cotyledons.
Figure 3.8 Average angle of cotyledon opening of WS (wild type) and gill under a range of conditions 
a) FR pulses (5 min/30 min)  
b) R pulses (5 min/30 min)  
c) R pulses (5 min/2h)  
Error bars represent the value for 95% confidence interval  
Under R pulses (5min/30min) gill displays altered cotyledon opening (b).  
However, normal cotyledon opening is observed under FR pulses (a) and  
less frequent R pulses (c).
3.2.3 Characterisation of the GIL1 gene

Co-segregation analysis suggests that the gill mutant phenotype is caused by a T-DNA (for T-DNA arrangement see figure 3.9) tagged gene. The T-DNA insertion occurs at a single locus. Additionally, co-segregation shows gill to be monogenic, and a recessive mutation. The presence of this T-DNA facilitates the use of a number of procedures to locate the gene position.

3.2.3.1 Mapping of GIL1

gill was crossed to the NW100 and to the NW80 mapping lines (Nottingham Arabidopsis Stock Centre) of Arabidopsis (Dr. Praekelt, pers com.). F2 seed was collected and screened for loss of any phenotypes associated with the mapping line. This provides information about the location of the gene affected. The segregation of the gill phenotype with the absence of a mapping line phenotype suggests that the defective gene responsible for the gill phenotype lies close to the gene for the lost mapping phenotype. If the gill mutation and the mapping line mutation are not linked then the expected frequency of the mapping line mutation is 1 in 4. If this ratio is different then it possible to calculate the distance of the gill mutation from this known gene. From the NW100 line, the gene causing the gill phenotype was mapped to chromosome V in the region of tt3 (transparent testa), approximately 10.4CM away. The msl (male sterility) mutation occurred at a ratio approaching 1 in 4, suggesting that the gill mutation occurs the opposite side of tt3. From the NW80 line gill was mapped to 26.8CM away from th2 (thiamine).
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Figure 3.9 Arrangement of pGBK5 T-DNA and position of primers for Genome Walking.
3.2.3.2 Genome walking

The knowledge of the sequence of the T-DNA allows the designing of primers that face out of the T-DNA towards the flanking DNA (figure 3.9). Digestion of the DNA with blunt ended restriction enzymes allows the ligation of adapters, of known sequence, to the DNA and hence PCR amplification from within the T-DNA to adapter primers (appendix 1). In this way it is possible to amplify a small section of the DNA next to the T-DNA insert. This is the method employed by the Clontech Genome Walker Kit. The original procedure is listed in section 2.3.6.

DNA was digested with Dra I (section 2.3.6) to check the purity of the DNA (figure 3.10). Lane 1 shows Dra I restricted DNA, lane 2 shows genomic DNA, the DNA is suitable for digestion. DNA was then digested individually with the supplied restriction enzymes, Eco RV, Sca I, Dra I, Stu I, Pvu II, and labelled DNA library 1 (DL1), DNA library 2 (DL2) etc. for the respective digests.

The DNA was purified (section 2.3.6) (figure 3.11), the bands were of equal intensity suggesting no DNA had been lost in the purification procedure. The Genome Walker adapter was ligated, and the procedure of amplification from the T-DNA performed.

To obtain successful results for the controls (figure 3.12), the procedure of genome walking was modified from that of the manufacturers' protocol (section 2.3.6). Further modification was required to obtain products for the genomic DNA restriction libraries. The result of this was a non-specific banding pattern using primers to the basta resistance gene within the T-DNA, but the reaction in lane 4 (figure 3.13b) consistently produced a bright band of approx. 2.6 kb (figure 3.14). The purified product and the appropriate primers (AP2 (adapter primer) and BAR2 (figure 3.9)) were sent to the automated sequencing service of Leicester University. Sequencing was achieved using fluorescent labelling.
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Figure 3.10 Genomic DNA digested with Dral (lane 1), and undigested genomic DNA (lane 2). This gel confirms that the genomic DNA is suitable for digestion, and hence genome walking.

Figure 3.11 Genomic DNA digested with various enzymes for Genome Walking. 
Lane 2 contains DNA before purification, this serves as a control to demonstrate that no DNA was lost during the purification step.
Figure 3.12 Genome Walking positive control PCRs

Lanes 2 and 3 show PCR products after primary PCR, a non-specific banding pattern is obtained. Secondary PCR uses nested primers in a second PCR reaction to obtain more specific amplification. This gel demonstrates that the genome walking protocol devised is suitable for amplification of products from positive controls.
Figure 3.13 Genome Walking PCR

a) Primary PCR from digested genomic DNA, lanes 1-5 correspond to different restriction digests (Eco RV, Sca I, Dra I, Stu I, Pvu II).

b) Secondary PCR, using product of primary PCR (numbers correspond to the samples in a). Amplification uses nested primers. In the secondary PCR a band occurs in lane 4, from the Stu I digested DNA, this band is approximately 2.5kb.

Figure 3.14 Repeated Genome Walking to obtain sample for sequencing.

Primary and secondary PCRs were repeated using Stu I digested sample. This demonstrated that the same sized fragment could be amplified on a separate occasion, and provided a product for sequencing.
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The initial sequence, by analysis in Sequence Navigator software, showed regions of sequence which were very A/T rich, with runs of thymine bases; these caused slippage of the sequencing and sequences of more than ~40 bases were not achieved. The high melting temperature (Tm) of the primers for effective genome walking were suboptimal for sequencing. For this reason the genome walker product (GWP) was cloned.

### 3.2.3.3 Cloning of the GenomeWalker product

To determine the sequence of the GWP it was necessary first to clone this fragment into a cloning vector for transformation of *E. coli*, which allows amplification of the product and facilitates PCR amplification with primers of sequencing compatible Tm.

The "Advantage Tth" Polymerase of the Universal GenomeWalker Kit has proof-reading properties, so all PCR products obtained are blunt ended; this restricts the choice of vector for cloning, and also reduces the chance of obtaining positive transformants as blunt ended cloning is less efficient than cloning using overhanging sequence. Two approaches to cloning were taken, one using the "Advantage Tth" amplified product in blunt ended cloning using pBS vector digested with Sma I. The second method used the same fragment amplified using Boehringer-Mannheim Expand™ High Fidelity PCR System, which contains 50% proof-reading ability and 50% standard Taq; this amplifies a mixed product of blunt ended and sticky ended DNA. The Expand Taq amplified product could be used with the blunt-ended cloning vector or with the A/T cloning vector. The vectors used were dephosphorylated to prevent re-ligation without the insert. The PCR amplification of samples for transformation was carried out with phosphorylated versions of the same primers, AP2 and BAR2, which allows ligation of the vector and insert.
Figure 3.15 Colony PCR for pBS containing the GWP.

pBS internal primers, T3 and T7, were used to PCR from colonies potentially containing the vector + insert. Lanes 2, 4, 5, 6, 7, 9 each contain a band at 2.5kb corresponding to the GWP size (figure 3.14). These samples therefore contain the insert and can be used for sequencing.
Figure 3.15b Sequence of cloned genome walker product.

Within the sequence obtained from the GWP the BAR2 primer sequence is evident, indicating that the expected fragment had been cloned. T-DNA sequence is present, sequence of Arabidopsis chromosome V continues from the end of T-DNA sequence.

Key
- BAR2 primer sequence
- T-DNA sequence
- Sequence with high homology to chromosome V of Arabidopsis
- Cloning vector
The cloning of the GWP into pBS vector was successful. Figure 3.15 shows the PCR products obtained from colony PCR of selected white colonies with T3 and T7 primers to the vector. Good quality sequence of 1 kb was obtained from 5 of these samples. The sequence contained matches to the AP2 and BAR2 primers that were internal from the vector primers. This confirmed that the correct GWP had been amplified and cloned, and that the product obtained was not due to any random priming of either AP2 or BAR2. Figure 3.15b shows the sequence obtained from this cloned fragment.

3.2.3.4 BLAST search for GWP sequence

The sequence obtained was entered into the BLAST (basic local alignment search tool) search on the internet (http://www.ncbi.nlm.nih.gov/BLAST/) for comparison with recognised nucleotide sequences.

The BLAST search recognised this fragment as between 91 and 100% identical to a sequenced region of the Arabidopsis chromosome V (figure 3.15b displays the T-DNA/chromosome V boundary). The TAC (transformation competent artificial chromosome) clone corresponding to the region is K19M22 (Kazusa database, http://www.kazusa.or.jp); this clone has been annotated to identify regions of potential genes / exons (figure 3.16a).

The DNA sequence next to the *gill* T-DNA insert corresponds to a region between predicted proteins 13 and 14 on the K19M22 clone (figure 3.16b).
Figure 3.16 a) Arrangement of clone K19M22 (Kazusa database)

b) Position of T-DNA within K19M22, from genome walking sequence, in relation to genes predicted by Kazusa database. The position of primers designed for further experiments are shown, each primer pair amplifies a region of 400-500bp (see appendix 1).
3.2.3.5 Confirmation of T-DNA position

The GWP was obtained by walking out from the T-DNA in one direction only. To confirm that the T-DNA is located between potential genes 13 and 14 on K19M22, and to establish whether the insertion has caused rearrangement or deletion of the DNA sequence in this region, PCR amplification from the opposite end (the end containing the GUS gene) of the T-DNA was performed.

Now that the sequence flanking the T-DNA was known it was possible to design primers to this sequence for use in combination with T-DNA primers. It had not been previously possible to amplify from the T-DNA in this direction. One reason for this may be the distance between the T-DNA primer and the adapter primer. If there are very few restriction sites within this area the fragments for amplification will be very large. Alternatively the sequence to which the T-DNA primers were designed at this end of the T-DNA could have been lost upon insertion of the T-DNA. Using primers to the sequence of chromosome V allows the prediction of fragment sizes and therefore enables optimum PCR conditions to be determined.

Primer 3-2 (figure 3.16b and appendix 1) to the genomic sequence of chromosome 5, ~400 bases downstream of the probable T-DNA insertion site was used to PCR amplify back to the T-DNA. Primers GUS5-1, GUS5-2, GUS5-3, GUS5-4 (to the T-DNA; figure 3.9 and appendix 1) were used in combination with primer 3-2. Figure 3.17 shows the PCR products obtained. For wt controls DNA from both WS and Col was used; the *gil1* mutant was isolated in the WS ecotype and the sequence database is constructed from Col; it was therefore important to include both of these as sequence variation between the ecotypes may occur, leading to amplification of different sized fragments. In the sample for GUS5-1 PCR products were obtained for Col and WS (figure 3.17, top gel, second primer pair) as well as for *gil1*, this would not be expected because there is no T-DNA insert in these samples. However, samples for the remaining primers all show obvious products for the *gil1* DNA and no product for Col or WS. The sizes of the bands obtained are consistent with the loss of some of the T-DNA right border. The products were sent for sequencing with the appropriate primers.
No bands are expected for wt (Col or WS) as the GUS primers are specific to the T-DNA. A number of different T-DNA primers were used as the T-DNA may not be complete, so the binding site for any one of these primers may be absent.

**Expected band size if T-DNA full-length:**
- 3-2 & GUS3-1: none unless T-DNA inverted
- 3-2 & GUS5-1: 1782bp
- 3-2 & GUS5-2: 1721bp
- 3-2 & GUS5-3: 1592bp
- 3-2 & GUS5-4: 2815bp

The bands observed on this gel are much smaller than those expected if the T-DNA is full-length therefore some of the T-DNA at the right border has been lost.
The sequence obtained again matched Arabidopsis chromosome V (the opposite side of the T-DNA to that obtained in the GWP); but this time the sequence from the T-DNA started 40 bases downstream, indicating that this small region was lost when the T-DNA inserted. The rest of the sequence matched that of the database, indicating that no major DNA rearrangement had occurred in this area. The sequence also confirmed that the first 594bp of the T-DNA right border were missing.

3.2.3.6 PCR of predicted genes in *gill*

T-DNA insertion can cause rearrangements of DNA and affect expression of genes some distance from the site of insertion. However, none of the neighbouring genes in *gill* display major rearrangements.

Primers were designed to the four predicted genes nearest to the site of T-DNA insertion (figure 3.16b and appendix 1). All primers were designed with the same Tm, and to amplify products of approximately the same size so that they could be used in different combinations and all primers could be used at the same time in one PCR machine.

PCR amplification of Col, WS and *gill* DNA was performed using primer pairs for the beginning of each gene (figure 3.18). The PCR cycles used were: 94°C 30 sec, 55°C 30 sec, 72°C 1 min for 30 cycles. No products were observed for any primer pair with WS DNA, and it must be assumed that this DNA was not suitable for PCR. Products of the expected size were observed in Col and *gill* for all primer pairs (figure 3.18), which indicates that the amplified regions, at least, are not altered in *gill*.
Region of K19M22 amplified by primer pairs, and expected size:
5-1 3-1 : region between K19M22.13 and T-DNA, 570bp
5-2 3-2 : K19M22.14, 410bp
5-3 3-3 : K19M22.13, 510bp
5-4 3-4 : K19M22.12, 590bp
5-5 3-5 : K19M22.15, 510bp

Figure 3.18  PCR amplification of regions of K19M22 to show that the T-DNA insert causes no major re-arrangements of the 4 nearest genes.
Bands of the expected size are observed in both Columbia and gill

b) Repeated PCR using primer pair 5-1 and 3-1
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Figure 3.19  PCR to confirm presence of T-DNA in *gill* between primers 5-1 and 3-2

The expected fragment size using primers 5-1 and 3-2 is 1509bp if no T-DNA is present. This size fragment was amplified in wt, but no fragment was amplified in *gill*, suggesting that the T-DNA is located between these two primers. In the same PCR a fragment of expected size, 410bp, was amplified from *gill* DNA using primers 3-2 and 5-2.
To confirm that the T-DNA is inserted in the proposed region (before confirmation by PCR amplification from T-DNA in both directions), PCR amplification (section 2.3.4.2) between primers 5-1 and 3-2 (figure 3.16b) was carried out using WS, Col and gill DNA. A product would be expected in the wt samples, without the T-DNA insert, and no product would be expected from gill DNA. Confirmation that the PCR was working was achieved using gill DNA with primer pair 5-2, 3-2 (figure 3.16b). The results of this experiment (figure 3.19) confirmed that the region between primers 5-1 and 3-2 could not be amplified in gill, but could be amplified in wt. From the same experiment it was possible to PCR amplify a different fragment of gill, showing that the PCR may have been effective were the T-DNA not present.

3.2.3.7 Screening a cDNA library for predicted protein 14

The nearest predicted gene to the site of T-DNA insertion is K19M22.14. Database searching suggests that this gene has no homology to any known existing gene. Although the next nearest gene is K19M22.13, a predicted protein kinase, it was decided to screen a cDNA library for gene 14. The close proximity of the T-DNA insert to this predicted gene suggested that if indeed it was expressed it would be affected by the T-DNA. The ATG of gene K19M22.14 is 524bp from the T-DNA insertion, whereas the ATG of gene K19M22.13 is 4359bp from the T-DNA insertion.

cDNA library screening was carried out as described in section 2.3.14. The probe was created by PCR between primers 5-2 and 3-2 (figure 3.16b), and the product was a 410 bp fragment of the predicted hypothetical protein. After three rounds of screening only one positive clone was identified in the cDNA library screen. A phage preparation was made from this clone (section 2.3.14) and was sequenced. The sequence was compared to the known sequence of K19M22.14 to confirm that the probe had identified the correct sequence. A small section of the clone did match the sequence of K19M22.14 exactly, confirming that this gene is expressed in wild type Columbia. However, the clone was chimeric, only a small section corresponded
to the required gene, the rest was unrecognisable sequence. In the construction of cDNA libraries it is possible for the creation of chimeras to occur. Within the exact match of the cDNA to the K19M22.14 sequence a region of ~40 bases was found to be missing from the cDNA. It could be speculated that this is a small intron, or it could be due to the construction of the cDNA. Further analysis was required for confirmation, however, the missing DNA was flanked by GT and AG, typical of intron-exon boundaries.

3.2.3.8 RT-PCR confirmation of hypothetical gene expression

RNA was extracted from rosette leaves of adult wild type and gill plants. First strand synthesis was carried out with primer 3-2 (figure 3.16b) to the hypothetical protein.

For RT-PCR two different primer combinations were used. Firstly primers 5-2 and 3-2 (figure 3.16b); if the hypothetical gene is expressed then a band should be obtained for wild type at least, but gill may not produce a band if the T-DNA insert is affecting the expression of this gene. Secondly the primers 5-1 and 3-2 (figure 3.16b) were used; by analysis of the database sequence it was observed that the predicted start codon for the gene K19M22.14 may not be the true start; two small open reading frames (ORF) were identified further upstream, the first of these also containing an ATG. Primer 5-1 lies within this first open reading frame, if these ORFs form part of the K19M22.14 gene, it would be expected that an RT-PCR product would be observed in wt, but not in gill, as the T-DNA would interrupt the sequence.

Figure 3.20 shows an agarose gel of the results from this experiment. Lanes 3&4, confirm the expression of the K19M22.14 protein, a band of the expected 400 bp size is observed; expression occurs not only in wt but also in gill. There are no obvious differences in expression levels between wt and gill, although the same starting quantity of RNA was used. Either the expression of this gene is not affected by the T-DNA insert in the gill mutant, or is affected only slightly and is
unresolvable by this method. The remaining lanes show no obvious band of the expected size for primers 5-1 and 3-2, so it is unlikely that the other ORFs observed form part of this gene.

Quantitative PCR was not performed. K19M22.14 appeared from the database to be a single exon; primers for RT-PCR would ideally span an intron, so that confirmation that DNA is not present and being amplified can be determined by the size of the product.
lanes 1&2: primers 5-1 and 3-2, expected product size approx. 650bp
lanes 3&4: primers 5-2 and 3-2, expected product size 410bp

Figure 3.20 RT-PCR gel.

Expected size product not observed in lanes 1 and 2, suggesting that the additional ORFs do not form part of the K19M22.14 gene.

Amplification of products of the expected size occurred in samples in lanes 3 and 4, suggesting that the K19M22.14 gene is expressed in both wt and gill.

PCR conditions must be optimised to prevent random priming and the PCR repeated to confirm results.
3.2.3.9 Northern blotting for detection of expression of the hypothetical gene

The expression of the hypothetical protein in wt and gill is evident from RT-PCR. Northern blotting allows the detection of different levels of expression of a gene. Total RNA, isolated using the Trizol method (section 2.3.1.2) was blotted as described in section 2.3.8. The blot was probed with same radiolabelled fragment as used in the detection of a cDNA clone (3.2.3.7). The blot was exposed to X-ray film for one week. No expression was detected after this time. The level of expression of the hypothetical gene must be very low in both wild type and gill.

The isolation of poly A+ RNA provides a pool of mRNA without the presence of ribosomal RNA that normally constitutes the majority of RNA isolated. Loading the same amount of mRNA as total RNA vastly increases the chance of detecting genes with low expression levels. RNA was isolated, using the Trizol method, from seedlings grown on agar plates for approx. 2-3 weeks. The concentration of RNA in the wt and gill samples was adjusted to the same level (125 µg in 200 µl). From these RNA samples, mRNA was extracted using the Clontech NucleoSpin kit (section 2.3.1.3). The concentration of the resulting mRNA was checked before electrophoresis. The probing of this blot was carried out using the Expresshyb system (section 2.3.8.2) with the same probe as for the cDNA library. The blot was exposed overnight on a PhosphorImager (Molecular Dynamics); quantification of the bands was achieved using Quantiscan software which calculates relative band intensity.

Figure 3.21 shows the RNA gel. Some ribosomal RNA was still present, which is apparent from the fairly intense banding pattern observed. Lane 5 has a lower level of ribosomal RNA present, it therefore would be expected that this sample contains more mRNA, as the same amount of total RNA was loaded. So, this sample may demonstrate a higher level of expression of the gene for the hypothetical protein.

The blot (figure 3.22) shows this to be the case, lane 5 shows higher expression than lane 4. The most comparable lanes in terms of RNA concentration (determined from the gel figure 3.21) are lanes 3 (wt) and 4 (gill), these suggest that the gene for the hypothetical protein may be expressed at a lower level in gill than in wt. This experiment would need to be repeated for confirmation of reduced K19M22.14 expression.
Figure 3.21 Gel for Northern blotting. WS and *gill* mRNA was isolated from 2-3 week old seedlings. Lane 5 appears to contain less ribosomal RNA, and therefore more mRNA.

Figure 3.22 Northern blot of gel above

The blot was probed with a radioactively labelled probe to K19M22.14. The fragment used was that amplified between primers 5-2 and 3-2 (figure 3.16b).

The band on this filter is approx. 1.5kb. This corresponds to the expected size of the K19M22.14 gene.
Quantification of the band intensity in Quantiscan estimates there to be a 2-fold reduction of expression in *gill*. Therefore it can be concluded that *gill* is not a null mutant but it does have reduced expression levels of a predicted gene.

The size of the transcript detected on the Northern blot was the same in *gill* as the wt, this suggests that the T-DNA insert is upstream of the start codon.

**3.2.3.10 Rapid Amplification of cDNA Ends (RACE) of *K19M22.14***

The start codon and the end of the *GIL1* gene are predicted in the database. The T-DNA is inserted 524 bases upstream of the ATG, and the gene is a single exon of predicted size 1451 bp, encoding 483 aa according to this information. It is necessary to confirm these positions, if there is another exon the start codon may be further upstream, placing the T-DNA in a translated region.

The Clontech 5' RACE kit was used to amplify both the 3' and 5' ends of the gene (section 2.3.7). Both total RNA and mRNA were used in this procedure, successful results were finally obtained using total RNA. The manufacturers protocol for first strand synthesis was followed. The cDNA was amplified with the appropriate 5' and 3' gene specific primers.

**3.2.3.10.1 3' RACE**

Using primers UP and GU5-8 (appendix 1) for 3' RACE a product was obtained of 1.6 kb (figure 3.23). PCR products of different sizes had been obtained previously using the same method, these products had proved unsuitable for sequencing directly. Cloned fragments revealed that the UP often bound at both ends. The 1 kb product also observed from the same PCR (figure 3.23), was a fragment amplified by the UP alone.
Figure 3.23 3'RACE PCR products using primers UP and 3-8. The 1.6kb band represents the 3'RACE product (determined by sequencing). The same size product was observed in *gill* and wt, suggesting that this section of K19M22.14 is unaffected by the T-DNA insert.
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Figure 3.24 Colony PCR, using primers M13 forward and M13 reverse, to confirm insertion of 1.6 kb 3'RACE product into vector
3'RACE was repeated for the 1.6 kb product, an additional 1 cycle of 72°C 10 min was used at the end of the previous cycles to increases the number of A overhangs for efficient cloning of the fragment.

In order to achieve more efficient transformation the TOPO® XL cloning kit from Invitrogen was used. This kit uses a vector with a lethal gene, insertion of a fragment disrupts this gene, so only positive transformants grow, kanamycin selection is also incorporated. Positive colonies were confirmed by PCR directly from the colony (section 2.3.9.8). Figure 3.24 shows the PCR products, amplified with M13 forward and reverse primers, from the 5 transformants selected, all of these are positive, containing a 1.6 kb fragment.

The sequence obtained corresponded with that of the K19M22.14 gene. An additional 114 bases were present at the 3’ end before the poly A tail, these correspond to the 3’ untranslated region.

### 3.2.3.10.2 5’ RACE

Primers 5-8 and UP (appendix 1) were used initially in the PCR but were unsuccessful in amplifying the correct product. A new primer was designed to match that of the universal primer mix (UPM) used in the Clontech 5’ RACE kit. PCR using the new UPM and primer 5-8 successfully amplified a 700 bp fragment (figure 3.25, lane 2). PCR using either primer alone resulted in a 1 kb band (lanes 3 and 4); this product could have masked the presence of the correct product in previous experiments. The 700 bp fragment from 5’ RACE was cloned and sequenced.

The sequence from the 5’ RACE product confirmed the predicted ATG to be the first start codon. The sequence obtained started 250 bases upstream of this. The T-DNA is located 524bp upstream of the ATG and therefore lies within the promoter region of the K19M22.14 gene.
Lane 2: 700bp PCR product obtained using primers UPM and 5RACEgil
Lane 3: 1 kb PCR product obtained using primer UPM alone
Lane 4: 1 kb PCR product obtained using primer 5RACEgil alone

Figure 3.25 5'RACE products

Lane 2 shows the 5'RACE fragment (determined by sequencing), the other lanes contain products of random priming by the individual primers.
3.2.3.11 Identification of an intron
As described in section 3.2.3.7 the sequence resulting from the cDNA suggested a small intron within the \textit{K19M22.14} gene. A 40 bp fragment was missing from the cDNA sequence in the 5' untranslated region, but was observed in sequence from DNA samples. The sequence of the 5' RACE product also lacks these 40 bases and therefore confirms that \textit{K19M22.14} does contain a small intron. The intron typically begins with GT and ends with AG (figure 3.26).

3.2.3.12 Protein analysis of K19M22.14
Analysis of the nucleotide sequence of \textit{K19M22.14} provides information about the possible protein form. \textit{K19M22.14} encodes a 54kDa protein predicted to have a high \(\alpha\)-helical content. Figure 3.27 shows the protein sequence.

Searching for recognised domains within this protein, and for similar proteins was largely unsuccessful. ProDom (Protein Domain- http://protein.toulouse.inra.fr/prodom/) recognised three domains as similar to domains in other proteins but none of these were functional. The most similar proteins recognised by PRINTS (Protein motif fingerprint database- http://www.biochem.ucl.ac.uk/bsm/dbbrowser/PRINTS/PRINTS/html) were myosin heavy chains and ryanodiners of Drosophila, however the similarity occurred between only a few amino acids and is unlikely to aid prediction of the role of K19M22.14. Propsearch uses amino acid composition, rather than the order of amino acids to search for similar proteins. The highest homology to K19M22.14 from this search was calcium independent protein kinases, however, no other searches recognised protein kinases so it is likely that the critical residues for protein kinase activity are not present in K19M22.14.

3.2.3.13 Overview of \textit{GIL1}
Results suggest that the \textit{gill} mutant phenotype arises from a T-DNA insertion in the promoter region of the \textit{K19M22.14} gene. This insertion leads to a reduced level of expression of this gene compared with wt. \textit{K19M22.14} contains a small intron of 40 bases and encodes a protein of 484 aa. Despite extensive database searching through
the internet, neither the DNA sequence nor the protein sequence show any homology to known genes.
Figure 3.26 Sequence of K19M22, showing the K19M22.14 region, the position of the 5' and 3' ends determined by RACE, the start codon, and the intron.
Figure 3.27 Protein sequence of K19M22.14
3.3 Discussion

A mutant has been isolated which defines an interesting link between light perception and gravitropism. *gill* is less randomised than wt under FR and R (continuous and pulses). However, some randomisation does still occur in *gill*, particularly under continuous FR, suggesting that *gill* retains some action of light-mediated randomisation. The *gill* mutant phenotype most likely arises due to a T-DNA insertion in the promoter region of a gene predicted by sequence analysis. Expression of this gene still occurs in *gill* showing that *gill* is not null.

The *gill* mutant does not display dramatically altered de-etiolation characteristics typical of existing mutants of the phyA or phyB pathways; the lesion appears to affect predominantly the light-mediated response to gravity. *gill* was isolated in a FR pulse screen, conditions under which phyA is the only active phytochrome. That *gill* shows reduced randomisation under such conditions implicates an impairment in the phyA control of gravitropism, at least through the VLFR.

Randomisation under high irradiance FR is also reduced in *gill*. However, wt and *gill* show much greater randomisation under HIR conditions than VLFR conditions. As *gill* retains some of its randomisation response under all conditions, it is possible that under high irradiance FR there is sufficient flux through the pathway to mediate an observable randomisation response to continuous FR. Alternatively the VLFR and HIR pathways for randomisation under FR could be genetically distinct, with GIL1 playing a greater role in the VLFR pathway. There is evidence that the VLFR and HIR exist as distinct pathways for some phyA mediated processes (Yanovsky *et al*, 1997 and chapter 4).

Under R, phyA is known to mediate hypocotyl randomisation through the VLFR, and phyB through the LFR (Poppe *et al*, 1996). Randomisation of wt hypocotyls under continuous R is slightly greater than under pulses of R. The randomisation of *gill* appears to be the same under both conditions, being less than wt (figure 3.5). Unlike FR, wt levels of randomisation are not induced in *gill* even under continuous...
R, so the *gill* phenotype is more prominent in R. Randomisation of *phyA* or *phyB* under continuous R is of the same order of magnitude as for wt. Only the double mutant *phyAphyB* shows impaired randomisation (Poppe *et al*, 1996); on this basis the reduced randomisation of *gill* observed in R would have to be due to impaired phyA and phyB pathways, as the impairment of either alone would not elicit a loss of randomisation. Therefore, it is possible that GIL1 defines a point in both the phyA and phyB pathways for gravitropic responses. GIL1 could mediate the loss of randomisation under FR pulses through phyA, and exert a greater gravitropic effect in R by action through two phytochrome pathways.

Reduced expression of the *K19M22.14* gene is observed in the *gill* mutant compared with wt (figure 3.22). Based on reduced levels of expression leading to the *gill* phenotype, there are two ways in which GIL1 could act. Firstly GIL1 could mediate an effect on the gravitropism pathway; with the loss of GIL1 the gravitropic effect is increased. This could only occur if GIL1 is a negative regulator of the gravitropism pathway. By reducing the amount of a negative regulator the gravitropism response would be increased. Secondly GIL1 could act either as a positive regulator of, or as an integral part of, a pathway which normally promotes the randomisation of hypocotyls. A decrease in the amount of GIL1 protein would then lead to less flux through this pathway and hence a reduction in the amount of randomisation.

The function of the randomisation of Arabidopsis hypocotyls remains unresolved. It has been observed that a R light pulse preceding a unilateral B light stimulus enhances both the first positive and second positive photocurvature. Both phyA and phyB play roles in phototropism as does at least one other phytochrome (Janoudi *et al*, 1997). The hypocotyl randomisation mediated by monochromatic R or even FR could provide an opportunity for the plant to perceive a phototropic stimulus from whichever direction it occurs. So it is not impossible that GIL1 plays a role in the enhancement of phototropism which occurs via the phytochrome pathways. The GIL1 protein could act as a negative or positive regulator as described above, this
time with enhancement of phototropic curvature or randomisation, rather than gravitropism, at the end of the phyA or phyB signalling pathway. In the presence of unidirectional B, hypocotyls bend to towards the light source, this bending is antagonised by responses to gravity. So a seedling bends towards the light, and then, in response to the gravity vector, straightens slightly, this is known as gravitropic compensation. To investigate whether GIL1 plays a role in gravitropic compensation, wt and gill seedlings could be grown in the presence of low fluence rate W from the side. The R will enhance phototropic curvature by disabling gravitropic compensation, and the B will stimulate phototropic curvature. Wt seedlings would be expected to bend towards the light source, if GIL1 plays a role in the enhancement of phototropic curvature then gill would be expected to display less bending towards the light source as this mutant still exhibits gravitropic compensation.

As all of the above possible roles of GIL1 are feasible, GIL1 could define an important point of crosstalk between the phytochrome and gravitropism pathways. It is unlikely that a single stimulus leads to a single response through one pathway alone. The reality is likely to be much more complex, with a network of pathways, probably with some common components, interacting to elicit a response.

From comparison of the K19M22.14 sequence with database entries, and from domain searching (e.g. ProDom http://www.toulouse.inra.fr/prodom.html) there is no evidence that K19M22.14 has any homology to any known genes, nor contains any recognised domains. Recent research has identified the presence of phyB in the nucleus (Sakamoto and Nagatani, 1996, Kircher, 1999). However, it is unlikely that K19M22.14 is localised in the nucleus as putative nuclear localisation signals (NLS) are not evident.

It can not be ruled out that GIL1 plays a role in the control of gravitropism by regulation of hormone levels. However, this is unexpected as the morphology of gill does not resemble any known Arabidopsis hormone mutants. If gill is a
hormone mutant then the hormone levels/sensitivity are altered only very slightly enabling wt plant morphology; or alteration of the hormone levels is occurring under certain conditions for limited time periods, enough to mediate the altered randomisation phenotype observed. Golan et al (1996) show that application of exogenous cytokinin can restore hypocotyl gravitropism under R. The same experiments applied to ethylene insensitive mutants show no restoration of the gravitropism under R. These experiments suggest that cytokinin acts by stimulating ethylene production to restore gravitropism in wild type. Based upon this evidence it could be argued that gill has amplified ethylene levels leading to constitutive gravitropism. The reduced expression of the GIL1 gene could only lead to increased synthesis of ethylene if GIL1 was a negative regulator of ethylene production. Alternatively gill could display amplified cytokinin levels and hence greater ethylene production.

Creation of gill ethylene insensitive double mutants would enable preliminary testing of this idea. As gill is not completely gravitropic under R or FR, it still remains possible to observe any restoration of gravitropism. If the double mutant was gravitropic then it would have to be assumed that the phenotype of gill is not due to an alteration in the response to ethylene; however if the double mutant was more randomised than gill, then it could be argued that gill overproduces ethylene (possibly via cytokinin) or increases the sensitivity of the hypocotyl to ethylene.

It is not expected that the gill phenotype results as an effect of ethylene. More randomisation of gill occurs in FR than R, so if gill constitutively overproduced ethylene then there would be an increased gravitropic response under continuous FR. However, if the ethylene production is not stimulated so strongly by FR then this greater randomisation response to FR could be explained.

3.4 Proposal of future GIL1 analysis
An important step in the future of the gill project is to confirm that the K19M22.14 gene is responsible for the mutant phenotype. The K19M22 clone is available from
the Ohio stock centre, and has been requested. However, the correct culture containing the K19M22 clone has not yet been received, confirmed by restriction digest of those clones which have been received. An alternative approach has been taken to obtain a fragment for complementation of the \textit{gill} mutant with the \textit{K19M22.14} gene.

A 4 kb fragment of DNA has been amplified by PCR using primers Eco 5-6 and Xba 3-6 (appendix 1). This contains the \textit{K19M22.14} gene and approximately 1.5 kb of upstream sequence and 1 kb of downstream sequence. This fragment has been subjected to a sequential restriction digest with \textit{Eco} RI and \textit{Xba} I (cut sites which were incorporated into the primer design). The Bluescript vector was simultaneously digested with the same enzymes. Transformation of bacteria has been successful, with the isolation of positive colonies (confirmed by PCR and restriction digest). The fragment was subcloned into Agrobacterium (Dr. Praekelt) and transformation of \textit{gill} is underway. Transformants can be selected on plates containing hygromycin, resistance to this is carried by the Agrobacterium, and can be screened under R and FR for the restoration of randomisation.

The \textit{gill} mutant is not null for the \textit{K19M22.14} gene, some expression still occurs, and some randomisation of hypocotyls can be observed particularly under continuous FR. The isolation of additional \textit{gil} alleles, particularly those which are null for the \textit{GIL1} gene will provide a more comprehensive picture for the function of \textit{GIL1}. Other phenotypes, not observed in \textit{gill} may arise, such as altered de­etiolation responses. The presence or absence of any additional phenotypes in a null mutant will determine whether \textit{GIL1} plays a role in gravitropism alone, or whether it has a much wider role in responses to light. It is proposed that EMS mutagenised Arabidopsis lines are screened for the \textit{gill} loss of randomisation phenotype to obtain null mutants, if successful these may provide greater insight to the structure and function of \textit{GIL1}. A parallel screen using the launch pad lines (http://www.jic.bbsrc.ac.uk) could provide more mutants. Launch pads are available at different locations within the Arabidopsis genome, a line which is close to the
gene of interest is selected. The position of *K19M22.14* on chromosome V is such that the launch pad line 398A is nearby. This line has been obtained and crossed to a transposase source producing F1 seed. The launch pad contains a Ds element which is activated by the transposase source. Simple crosses of the two lines can be made without the need for emasculation of the buds, as true crosses can be selected by herbicide resistance. The F1 seed can be subjected to selection with the herbicide basta, those which are resistant are true crosses. The F2 seed can be selected on cholsulfuron for the excision of the transposon. The F3 seed is then ready for screening in the plate rotation assay for the *gill* phenotype.

Further physiological characterisation of *gill* and other alleles isolated would provide valuable information concerning the role of GIL1. Of particular interest is the possible interaction of GIL1 with phototropism. Traditional experiments using a brief R light pulse followed by unilateral B (Janoudi and Poff, 1992) will determine any effects of the *gill* mutation on the first or second positive photocurvature. Additionally the adult phenotype should be studied in detail. No obvious alterations to the pattern of growth have been observed in *gill* during growth in greenhouse conditions. However, it is possible that null mutants and perhaps *gill* will demonstrate alterations in other gravity controlled responses, such as branch angle and leaf angle, under more controlled conditions.

Database searching has not yielded any genes with regions of homology to *K19M22.14*. As the Arabidopsis genome project advances regular searches should be made to identify any other genes with related functions. In addition, a low stringency Southern analysis could be used to identify the presence of other gene family members, which could in turn be tested for complementation of the *gill* phenotype. Arabidopsis is not the only plant to display randomisation of hypocotyl growth under monochromatic light conditions. Orthologs of the *GIL1* gene may exist in these plants with the same function, and may also exist in other plant species if the role of GIL1 is more than that of transient randomisation. This possibility could be tested using DNA hybridisation.
The expression pattern of *GIL1* within a plant may yield much more information about its role. Expression may be maximal in seedlings, particularly in the hypocotyl cells which respond to the gravitropic stimulus (although some expression occurs in adult plants, determined by the isolation of a cDNA clone from a cDNA library created from rosette leaves, and by RT-PCR from adult plant tissue). To investigate expression patterns northern blotting and reporter gene analysis can be implemented. It would also be possible to determine the localisation of GIL1 within the cell using GFP fusion analysis. Screening of seedlings, which overexpress *GIL1* under the control of the CaMV 35S promoter, in comparison with null mutants, will provide more information concerning the role of GIL1.

GIL1 forms part of the light-mediated randomisation interaction, and so provides a point of crosstalk between phytochrome pathways and gravitropism, as such GIL1 may interact with a number of proteins. Other proteins which interact with phytochrome directly such as PIF3 (Ni et al., 1998) and PKS1 (Fankhauser et al., 1999) have been identified using the yeast two-hybrid assay. The same approach could be utilised to identify factors with which GIL1 interacts. Mutants corresponding to any novel interacting factors could be isolated by screening the SLAT filters. The SLAT filters contain inverse PCR products from pools of transposants, once a positive pool has been identified plants from that pool can be screened to find the mutant.
4.0 \textit{fhy3} - A Mutant of the phyA Signal Transduction Pathway Displays Amplified Responses to R.

4.1 Introduction

Of the five Arabidopsis phytochromes, the signalling pathway of phyA is perhaps the best characterised. The isolation of \textit{phyA} mutants, and of phyA signalling mutants especially, has contributed greatly to our current understanding of phyA mediated responses.

The mutants \textit{fhy1} and \textit{fhy3} (Whitelam et al, 1993) were the first phyA signalling mutants identified. These mutants displayed a reduced response to FR for inhibition of hypocotyl elongation and cotyledon expansion, but were not deficient in photoactive phyA. The mutants defined different complementation groups and were distinct from \textit{phyA}; this suggested that \textit{fhy1} and \textit{fhy3} defined signalling components of the phyA pathway. Since the onset of this study other phyA signalling mutants have been reported such as \textit{spal} (Hoecker et al, 1998), and \textit{far1} (Hudson et al, 1999). Mutants affecting specifically the phyB pathway have also been identified, e.g. \textit{red1} (Wagner et al, 1997) and \textit{pef2} and \textit{pef3} (Ahmad and Cashmore, 1996). Mutants which display aberrant responses mediated by a particular phytochrome lead us to believe that there are components specific to individual phytochrome pathways. However, there is also evidence that interaction between phytochrome pathways exists and that some proteins do not act in one phytochrome pathway alone. The mutant \textit{psi2} (phytochrome signalling 2) leads to amplified responses to both FR and R through the phyA and phyB pathways respectively (Genoud et al, 1998). Identification of PIF3 through both the \textit{pocl} mutant (Halliday et al, 1999), and using yeast two-hybrid screening (Ni et al, 1998), has demonstrated that factors exist that can interact with more than one phytochrome.

Interaction between the phyA and phyB signalling pathways is known to occur. Under R, phyB mediates de-etiolation through a LFR; however, partial de-etiolation
also occurs through a phyA VLFR (Mazzella et al, 1997). In wild type plants a second phyA response is the negative regulation of the phyB LFR. A FR pretreatment enhances the LFR induced by a R pulse (Cerdán et al, 1999). So, phyA regulates the phyB LFR in either a negative or a positive manner dependent upon light conditions. Both of these interactions require FHY1 (Cerdán et al, 1999). The possible roles of FHY3 in the action of phyA mediated R responses, and also of phyB mediated responses will be discussed in this chapter.

4.2 Results

New alleles of the fhy3 mutation have now been isolated in different ecotypes of Arabidopsis. Those available during the time of this project, and their respective ecotypes, are listed below with the research group that provided them:

Columbia - fhy3-1 (Whitelam)
Columbia - cf3271, cf3231 (Harberd)
WS - fhy3 (Whitelam)
Landsberg erecta - ifn261, ifn282, ifn223 (Harberd)

All of the new mutant alleles were isolated from screens to identify mutants which displayed a long hypocotyl under FR, and were shown in allelism tests to be fhy3.

4.2.1 Physiological analysis of fhy3

4.2.1.1 The response of fhy3 to FR

Each of the fhy3 alleles was grown with its respective wild type under 3d continuous FR to verify the reduced inhibition of hypocotyl elongation in these mutants, and hence a role in the phyA signal transduction pathway (figure 4.1). Seeds were germinated as described in section 2.1.2, following the final dark period plates were transferred to continuous FR. All fhy3 alleles displayed a long hypocotyl compared with wt confirming that phyA signalling was indeed impaired in these mutants. There is a difference in the degree of response to FR by the different alleles.
Figure 4.1 Wild types (Landsberg *erecta* (Laer), Columbia (Col-O), and Wassilewskija WS)) and the respective *fhy3* alleles, grown under 3d continuous FR. *fhy3-1* was the original allele isolated.

*fhy3* displays reduced de-etiolation responses to FR.
4.2.1.2 Fluence rate dependency of fhy3 inhibition of hypocotyl elongation

In fhy3-1 some residual phyA action still exists so the inhibition of hypocotyl elongation, as with wild type, is fluence rate dependent. The lengths of hypocotyls for all of the fhy3 alleles and their wts were measured.

Figure 4.2 shows the hypocotyl length of Col and Col fhy3 alleles grown under continuous FR of different fluence rates. The long hypocotyl phenotype of fhy3 is apparent at fluence rates greater than 0.1 μmol m$^{-2}$ s$^{-1}$. However, all Col fhy3 alleles display a fluence-rate-dependent inhibition of hypocotyl elongation, with greater inhibition being observed at higher fluence rates. Figures 4.3 and 4.4 show that Laer and WS fhy3 alleles display the same fluence-rate-dependent inhibition of hypocotyl elongation, and that this inhibition is reduced compared with that of wt over a range of fluence rates. The inhibition of hypocotyl elongation of Col fhy3-1 and WS fhy3 does not increase greatly at fluence rates higher than 1 μmol m$^{-2}$ s$^{-1}$, whereas the other fhy3 alleles display significant inhibition of hypocotyl elongation at these fluence rates.
Figure 4.2 Average hypocotyl length of 30 seedlings for Col and Col \( fhy3 \) alleles under 3d continuous FR at different fluence rates.

\( fhy3 \) alleles have longer hypocotyls than wt, and therefore display reduced de-etiolation under these conditions.

Error bars represent value for 95% confidence interval.
Figure 4.3 Average hypocotyl length of 30 seedlings for Laer and Ifn alleles under 3d continuous FR at different fluence rates. 

Ifn alleles have longer hypocotyls than wt, and therefore display reduced de- etiolation under these conditions.

Error bars represent value for 95% confidence interval.
Figure 4.4 Average hypocotyl length of WS and WS fhy3 under 3d continuous FR at different fluence rates.

WS fhy3 has longer hypocotyls than wt, and therefore displays reduced de-etiolation under these conditions.

Error bars represent value for 95% confidence interval
4.2.1.3 Phenotype of seedlings exposed to R

The \textit{fhy3-l} mutant demonstrates an enhanced inhibition of hypocotyl elongation in response to continuous R (Whitelam \textit{et al}, 1993). This is an interesting phenotype, suggesting a role for phyA in R or perhaps that FHY3 is also involved in phyB signalling. This amplified response to R is here characterised in more detail.

4.2.1.4 Hypocotyl phenotype of \textit{fhy3} in R

The paper describing \textit{phyA}, \textit{fliyl} and \textit{fhy3} (Whitelam \textit{et al}, 1993) shows reduced hypocotyl length of \textit{fhy3} under continuous R; i.e. \textit{fhy3} shows enhanced inhibition of hypocotyl elongation compared to wt. This response could be mediated by an increase in sensitivity to all fluence rates of R, or could be due to a shift in the sensitivity to R. In other words, the curve for inhibition of hypocotyl elongation through increasing fluence rates of R light could be shifted to the left, so producing at low fluence rates a response normally observed at a high fluence rate. The hypocotyl lengths of the different \textit{fhy3} alleles and wts were measured over a range of R fluence rates (figures 4.5, 4.6, 4.7).

The results of this experiment demonstrate that \textit{fhy3} retains a fluence-rate-dependent response to R. At higher fluence rates of R the inhibition of hypocotyl elongation is greater than that at lower fluence rates. This is true for wt and all \textit{fhy3} alleles. However, not all of the \textit{fhy3} alleles demonstrate a greater inhibition of hypocotyl elongation compared to wt. Ws \textit{fhy3} and Col \textit{fhy3-l} display an obvious enhanced R response for hypocotyl elongation (figures 4.5 and 4.7). The Laer \textit{lfn223} allele also displays greater hypocotyl inhibition than wt in this experiment (figure 4.6). Although no obvious enhanced response was observed for this allele when grown for other experiments. There is little or no effect of the \textit{fhy3} mutation on the inhibition of hypocotyl elongation, under R, in the remaining Col and Landsberg alleles. These results could indicate allelic differences between the \textit{fhy3} mutations or could equally be ecotype specific variations.
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*fhy3*-A Mutant of the phyA Signal Transduction Pathway Displays Amplified Responses to R

**Figure 4.5** Average hypocotyl length of 30 seedlings for Col and Col *fhy3* alleles under 3d continuous R at different fluence rates.

Col *fhy3*-1 displays an amplified response to R for inhibition of hypocotyl elongation. This response is not evident in the other Col *fhy3* alleles.

Error bars represent value for 95% confidence interval.
Figure 4.6 Average hypocotyl length of 30 seedlings for Laer and /fn alleles under 3d continuous R at different fluence rates.

Ifn223 displays an amplified response to R for inhibition of hypocotyl elongation. This response is not so apparent in other Laer fhy3 alleles.

Error bars represent value for 95% confidence interval.
Figure 4.7 Average hypocotyl length of 30 seedlings for WS and WS fhy3 under 3d continuous R at different fluence rates.

WS fhy3 displays an amplified response for inhibition of hypocotyl elongation.

Error bars represent value for 95% confidence interval
4.2.1.5 Cotyledon phenotype of \textit{fhy3} in R

The data for hypocotyl elongation under R show amplified responses in only some alleles. However, \textit{fhy3} demonstrates amplified R responses for other de-etiolation processes. R, in the stimulation of de-etiolation, inhibits hypocotyl elongation and promotes hook opening and cotyledon expansion. In order to determine the degree of increased sensitivity of the different \textit{fhy3} alleles to R, the expansion of cotyledons was measured after 3d continuous R (figures 4.8 and 4.9). Again this experiment was carried out at different fluence rates of R as any enhanced responses may be more apparent at a particular fluence rate.

In contrast to the experiment to investigate inhibition of hypocotyl elongation, where only two \textit{fhy3} alleles showed any variation from the wt, this experiment demonstrates an amplified response to R in terms of cotyledon expansion for all of the \textit{fhy3} alleles. Larger cotyledons are observed on all \textit{fhy3} alleles compared with their wt, although the degree of enlargement varies. Landsberg alleles demonstrate a very slight increase in cotyledon size, which is nevertheless observable, whereas Col and WS \textit{fhy3} alleles display a much greater enhancement of cotyledon expansion. So the amplification of responses to R is not limited to particular alleles/ecotypes, although the manifestation of that amplified signal varies in an ecotypic/allelic manner.
Figure 4.8 Mean cotyledon area of 30 seedlings for Col and Col $fhy3$ alleles under 3d continuous R at different fluence rates.

The cotyledon area of Col $fhy3$ alleles is greater than that of wt, therefore this de-etiolation response to R is amplified in $fhy3$.

Error bars represent value for 95% confidence interval.
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Figure 4.9 Mean cotyledon area of 30 seedlings for Laer and WS wild types and *fhy3* alleles, under 3d continuous R at different fluence rates.

The cotyledon area of the *fhy3* alleles is greater than that of wt, therefore this de-etiolation response to R is amplified in *fhy3*.

Error bars represent value for 95% confidence interval.
4.2.1.6 Double mutant analysis

The action of FHY3 in R could occur through either phyA or phyB to mediate those responses observed. The phyAphyB double mutant exhibits a hypocotyl length greater than that of phyB alone under R, and therefore phyA plays a role in de-etiolation under R. Hence, analysis of the double mutant fhy3phyB may provide a clue concerning the action of FHY3 in R. If, under R, the fhy3phyB double mutant were shorter than the phyB mutant, then the enhanced de-etiolation of fhy3 could be said to be mediated by phyA as this action will still be evident. However, if the fhy3phyB double mutant hypocotyl is longer than that of phyB then the R light associated phenotypes of some fhy3 mutants must be dependent upon phyB.

Those alleles that displayed the most exaggerated response under R (WS fhy3 and Col fhy3-1) were selected for crossing. Each of these fhy3 alleles was crossed with phyB (phyB-9 of Columbia which is null, or a tagged phyB allele from Feldman lines for WS) and the F2 screened under FR light for long hypocotyls, those seedlings selected are all homozygous for the fhy3 mutation. Individual plants were then screened under R alongside phyB for 3d (figure 4.10). The Col fhy3phyB double mutant displays an elongated hypocotyl compared with phyB. For WS the seedling chosen was heterozygous for phyB, Mendelian segregation of 3:1 short:long was observed. The hypocotyls which are long are true fhy3phyB doubles. These hypocotyls are longer than those of monogenic phyB. So, the data suggest a role for phyB in the amplified responses to R observed in some fhy3 mutants.
Figure 4.10 Screening under R for *fhy3phyB* double mutants

a) Col *fhy3phyB* double seedlings are slightly longer, as a population, than the Col *phyB* mutant under continuous R.

b) WS *fhy3*, heterozygous for *phyB* (F3 seeds). The *fhy3phyB* double mutant seedlings (top two rows) were slightly longer than WS *phyB* under continuous R.
4.2.2 \textit{FHY3} and gene expression

\textit{fhy1-1} which was isolated in the same screen as \textit{fhy3-1}, and also defines a phyA signalling component, was shown to exhibit a block in the regulation of \textit{CHS} and \textit{CHI} (Barnes \textit{et al}, 1996). Expression of \textit{PC} and \textit{RBCS} was also shown to be drastically reduced in \textit{fhy1-1}. However, the genes \textit{CAB} and \textit{NR} were not dramatically affected in \textit{fhy1-1}; the induction of all of these genes is blocked in \textit{phyA}, so \textit{FHY1} is involved in only some phyA responses and thus defines a branch point in phyA signalling. To determine the role of \textit{FHY3} in the phyA signalling pathway expression of the genes mentioned above was investigated using northern blotting. This work was carried out by Dr. P Devlin (Scripps Institute).

Gene expression in Col \textit{fhy3-1} was measured at a number of time points for both FR and R. Figure 4.11 shows \textit{CAB} and \textit{CHS} expression in FR and R. \textit{CHS} levels are unaffected in the \textit{fhy3-1} mutant in both FR and R. However, \textit{CAB} expression in \textit{fhy3-1} under FR may be slightly reduced, and under R an obvious reduction in amplitude of \textit{CAB} cycling is observed (figure 4.11). \textit{PC} and \textit{RBCS} expression (figure 4.12) is induced slightly less in \textit{fhy3-1}. None of the genes have enhanced expression under R in \textit{fhy3-1}. The expression of the described genes in \textit{fhy3} is unaffected in the first six hours of light treatment, this suggests that the VLFR is functional in \textit{fhy3}. This pattern of gene expression contrasts with that observed in \textit{fhy1}. \textit{fhy3} has altered \textit{CAB} expression but unaffected \textit{CHS} expression, whereas \textit{fhy1} has altered \textit{CHS} expression but unaffected \textit{CAB} expression. This result may interestingly place \textit{FHY1} and \textit{FHY3} in different signalling pathways both from phyA. Expression of \textit{CAB} and \textit{CHS} has been proposed to be mediated different pathways; \textit{Ca}\textsuperscript{2+}/calmodulin and the cGMP pathways respectively (Bowler \textit{et al}, 1994). So, with these pathways in mind it could be speculated that \textit{FHY1} affects the cGMP pathway, whereas \textit{FHY3} affects the \textit{Ca}\textsuperscript{2+}/calmodulin pathway.
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Figure 4.11 Expression of CAB and CHS in Col and *fhy3*-1 under FR and R.
Under R there is a decrease in amplitude for CAB expression in *fhy3*-1.

Time (h): time elapsed since transfer to FR or R.
Error bars represent standard deviation.

(data courtesy of Dr. P. Devlin, Scripps Institute)
Figure 4.12 Expression of PC and RBCS in Col and fhy3-1 under FR and R. Under FR RBCS expression is reduced in fhy3-1.

Time (h): time elapsed since transfer to FR or R.
Error bars represent standard deviation.

(data courtesy of Dr. P. Devlin, Scripps Institute)
4.3 Discussion

The mutant \textit{fhy3} is impaired in phyA signalling. Reduced de-etiolation occurs in \textit{fhy3} under FR. However, an enhancement of R mediated de-etiolation occurs in \textit{fhy3}. These enhanced responses to R require the phyB pathway, demonstrated by the analysis of \textit{fhy3phyB} double mutants. FHY3, may in contrast to FHY1, play a role in the Ca\(^{2+}\)/calmodulin pathway and not in the cGMP pathway, suggested by expression analysis of genes predicted to be regulated by these pathways. The normal gene expression in \textit{fhy3} for the first six hours (figures 4.11 and 4.12), coupled with the observation that the reduced inhibition of hypocotyl elongation under FR is not apparent at very low fluence rates (figures 4.2, 4.3 and 4.4), suggests that \textit{fhy3} may retain a normal VLFR but be impaired in the HIR.

The \textit{fhy3} mutant displays a reduced inhibition of hypocotyl elongation by FR (figures 4.2, 4.3, 4.4 and Whitelam \textit{et al}, 1993). Over the range of fluence rates used the phenotype of \textit{fhy3} is more prominent at the higher fluence rates, above 1.0 \(\mu\text{mol}\) m\(^{-2}\) s\(^{-1}\). The similarity between \textit{fhy3} and wt at the lowest fluence rates suggests a relatively normal VLFR in these mutants. The altered de-etiolation response becomes evident at fluence rates above 1.0 \(\mu\text{mol}\) m\(^{-2}\) s\(^{-1}\), leading to the proposal that \textit{fhy3} is lacking the HIR. In fact \textit{fhy3} does not completely lack the HIR but the responses are shifted to higher fluence rates. The evidence for this comes from the comparison of hourly and continuous FR of 180 mol m\(^{-2}\) h\(^{-1}\) for the de-etiolation responses of hypocotyl elongation and cotyledon opening in wt and \textit{fhy3-1} introgressed into Landsberg \textit{erecta} (Yanovsky \textit{et al}, pers. com. submitted). Continuous FR still caused greater de-etiolation responses in \textit{fhy3} than was evident under pulses of FR.

In \textit{fhy3}, pulses of FR are sufficient to increase seed germination above that of dark controls, this response is lacking in the \textit{phyA} mutant (Yanovsky \textit{et al}, pers. com. submitted). So phyA mediated control of seed germination is not affected in \textit{fhy3}, providing further evidence to support the presence of an active VLFR in \textit{fhy3}.
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The Col *fhy3*-1 allele demonstrates an amplified de-etiolation response in R for both hypocotyl inhibition (Whitelam *et al.*, 1993 and figure 4.5) and cotyledon expansion (figure 4.8). The WS *fhy3* allele shows a comparable phenotype (figures 4.7 and 4.9). The more recently obtained Col and Laer alleles do not demonstrate such a prominent R phenotype, and hypocotyl inhibition is very similar to that of wt in all except *ln 223* which does display a short hypocotyl. Cotyledon expansion in these newer alleles is also less dramatic but is nevertheless still apparent. Consistent with these results Col *fhy3*-1, under continuous R, displayed enhanced inhibition of hypocotyl elongation and greater cotyledon unfolding; however, *fhy3* introgressed into Laer did not obviously enhance R responses (Yanovsky pers. com.). It was also observed that the amplified response of *fhy3*-1 to R was less prominent under pulses of R than under continuous R (Yanovsky pers. com.). So at least some *fhy3* alleles have amplified responses to continuous R.

The majority of the de-etiolation response observed in wt under R is mediated by phyB. However, phyA is active in R and plays a number of roles. Firstly phyA can mediate an enhancement of phototropic curvature through the perception of a R pulse (chapter 3). More relevant to this investigation, phyA detects very low fluences of R to mediate inhibition of hypocotyl elongation (Mazzella *et al.*, 1997). However, analysis of the *phyA* mutant reveals a negative effect of phyA on the LFR mediated by phyB; phyA mutants have greater inhibition of hypocotyl elongation under high fluence rates of R (Mazzella *et al.*, 1997). Additionally phyA and phyB can act synergistically; pre-irradiation with FR, followed by a pulse of R leads to amplification of the responses mediated by the phyB action. The cumulative effect of these responses leads to the lack of phenotype for the *phyA* mutant under low fluence rate continuous R.

The amplified response of the *fhy3* mutant to R could be explained by the action of phyA or phyB. As the *fhy3* mutant has impaired FR responses and therefore plays a role in the phyA pathway it is tempting to explain the amplified R phenotype through the action of phyA. *fhy3* displays no reduction in the levels of
immunodetectable phyA (Whitelam et al., 1993); however, some laboratories have reported the observation of a slightly increased level of phyA (Fairchild et al., 1996). If indeed a greater phyA action occurred it could be argued that the phyA VLFR, which occurs in R to inhibit hypocotyl elongation, is enhanced leading to a more pronounced de-etiolation in fhy3. However, there would presumably be greater negative effect on the phyB LFR which counteracts the inhibition of hypocotyl elongation. Increased action of phyA is not consistent with the FR phenotype of fhy3, explanation of this is only viable if the VLFR and HIR could be separated, with the loss of FHY3 affecting only one of these. Of course, this is a possibility, supported by the identification of the VLF loci (Yanovsky et al., 1997). The VLF loci are involved in some phyA responses (those of the VLFR) without affecting responses apparently mediated through the LFR or HIR. So phyA may act through two separable pathways. Evidence reported above suggests that fhy3 retains a normal VLFR but has a reduced HIR in FR, the hypocotyl inhibition being apparently normal at very low fluence rates. However, there is no evidence for an enhanced VLFR in FR, as hypocotyl elongation is comparable to wt. Enhancement of the phyA VLFR in fhy3 under R to mediate the increased de-etiolation therefore seems unlikely, unless the phyA mediated VLFR acts differently in R to that in FR.

Alternative explanations of the fhy3 phenotype under R exist. Figure 4.13 shows models of possible FHY3 action. As proposed in figure 4.13a FHY3 could exist as a regulator of both the phyA and phyB pathways, being an integral part of neither. In this case FHY3 could regulate the two pathways in an opposing manner. In the fhy3 mutant positive regulation of phyA hypocotyl inhibition would be lost leading to longer hypocotyls in FR. In R the fhy3 mutant would display shorter hypocotyls if a negative regulation of the phyB pathway by FHY3 was lost.

FHY3 could form an integral component of the phyA pathway and still exert a negative effect on inhibition of hypocotyl elongation by phyB (figure 4.13b). The loss of FHY3 in this case would lead to the same responses described above. The full action of phyA in FR would require the presence of FHY3. Inhibition of
hypocotyl elongation under R would be regulated in a negative manner in the presence of FHY3 in the active phyA pathway. Loss of FHY3 in the mutant would remove the negative effect leading to enhancement of inhibition of hypocotyl elongation.

In the third proposal (figure 4.13c) FHY3 forms an integral part of both the phyA and phyB pathway. In this scenario FHY3 is required for the action of phyA inhibition of hypocotyl elongation under FR. In R the role of FHY3 in the phyB pathway could be antagonistic to inhibition of hypocotyl elongation, therefore the fhy3 mutant would have shorter hypocotyls. This pathway proposes that phyA and phyB pathways for inhibition of hypocotyl elongation are separate after FHY3, rather than FHY3 being a branch point for the merging of the two pathways to control inhibition of hypocotyl elongation.

Figure 4.13 Proposed roles of FHY3 in phytochrome A and phytochrome B signal transduction pathways.
The fact that the double mutant *fhy3*-*phyB* has longer hypocotyls under R than monogenic *phyB* leads to the proposal that the enhanced inhibition in *fhy3* under R depends upon phyB (section 4.2.1.6). This discounts the idea that the amplified R response of *fhy3* is caused by increased phyA action directly to inhibit hypocotyl elongation. However, any of the described actions above (figure 4.13) of FHY3 to regulate the phyB pathway remain viable.

The role of phyA in R to regulate phyB action is now well documented (Mazzella *et al.*, 1997, Cerdan *et al.*, 1999), and FHY1 has been shown to play a role in both the antagonistic and synergistic action of phyA in R (Cerdán *et al.*, 1999). This provides evidence that a pathway such as that proposed in figure 4.13b could exist. So the most likely action of FHY3 in R is through phyA regulation of the phyB pathway (figure 4.13b).

The difference in responses to R of the *fhy3* mutants could be explained by the different alleles or by the ecotype. All Laer *fhy3* alleles have a less pronounced phenotype in R than the *fhy3* alleles of other ecotypes. This is true for the data presented in this thesis and for the *fhy3*-l allele introgressed into Landsberg *erecta* (Yanovsky pers. com.). However, the phenotype in FR relates directly to the phenotype in R. Figure 4.1 shows hypocotyl length of the different *fhy3* alleles. Col *fhy3* and WS *fhy3* alleles display a greater loss of inhibition of hypocotyl elongation than Laer alleles. When comparing this observation with those of de-etiolation in R (figures 4.5-4.9), it can be observed that those alleles that have the most prominent FR phenotype are the same alleles as those with the most enhanced R de-etiolation. So the dependence of the R phenotype on either ecotype or allele can not be resolved conclusively.

The ecotype Columbia is known to have a reduced VLFR compared to Laer (Yanovsky *et al.*, 1997). The strength of the WS VLFR is unknown in relation to either of these two ecotypes. The reduced VLFR of Columbia means that phyA action under R, to inhibit hypocotyl elongation, may be much less than that
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occurring in Laer. However, the antagonistic action of phyA with phyB, to prevent the complete de-etiolation of hypocotyls by phyB, could still be occurring in Columbia. Thus, if FHY3 plays a role in this antagonistic action, the fhy3 mutant will appear shorter than wt. In Laer both the VLFR to inhibit hypocotyl elongation and the antagonistic action of phyA on phyB are present. The VLFR of Laer is much stronger than that of Col, therefore this response may be displayed by the Laer wt seedling to a greater extent than is displayed in the Col wt seedling. Removing FHY3 which is acting through the antagonistic pathway may not lead to an obvious effect in Laer because the hypocotyl already has a greater degree of de-etiolation mediated through the direct action of phyA in R. In other words the Laer hypocotyl is already short, whereas Col has a greater capacity remaining for further de-etiolation of the hypocotyl.

This work demonstrates that FHY3 is an interesting component of the phyA pathway. Similarly to FHY1, FHY3 plays a role in both FR and R mediated responses, particularly for inhibition of hypocotyl elongation and cotyledon expansion. However, the mode of action of FHY3 is may differ from that of FHY1. FHY3 responses appear to be mediated through the proposed Ca$^{2+}$/calmodulin pathway, and predominantly occur via the HIR. FHY1, on the other hand, appears to mediate responses through the proposed cGMP pathway, predominantly through the VLFR. This occurs despite the role of both FHY1 and FHY3 in FR being to enhance de-etiolation processes through phyA. Both FHY1 and FHY3 define interesting points of crosstalk between phyA and phyB.
5.0 Putative Suppressors of \textit{fhyl-1} (soft)

5.1 Introduction

Screening for mutants affected in the phyA signal transduction pathway, by isolating mutants that display a long hypocotyl in FR has been fairly comprehensive. However, an alternative approach to identify novel components is to screen for the suppression of a known mutation. This method has successfully identified suppressors of \textit{phyB} (Reed et al., 1998), a suppressor of \textit{phyA} (spa1) (Hoecker et al., 1998), and suppressors of \textit{hy2} (a chromophore deficient mutant) (Kim et al., 1996). The aim of this project was to identify new components of the phyA signalling pathway by isolation of mutants demonstrating a suppression of the \textit{fhyl} phenotype.

The \textit{fhyl} mutant was identified at the same time as \textit{phyA} and \textit{fhy3} by Whitelam et al. (1993). This mutant displayed a long hypocotyl in FR, but defined a separate genetic locus to \textit{phyA} or \textit{fhy3}. In fact \textit{fhyl}, like \textit{fhy3}, retains some response to FR in terms of inhibition of hypocotyl elongation. This suggests either that all of the alleles isolated for both of these mutations are not null, or that \textit{fhyl} and \textit{fhy3} define different branches of phyA signalling.

There is evidence from analysis of gene expression that \textit{fhyl} defines a branch point in phyA signalling (Barnes et al., 1996). The induction of the genes \textit{CHI}, \textit{CHS}, \textit{PC} and \textit{RBCS} under continuous FR is blocked in \textit{fhyl-1}, whereas the induction of \textit{CAB} and \textit{NR} is nearly normal; the induction of expression of all of these genes under FR is blocked in the \textit{phyA} mutant. This suggests that \textit{fhyl} defines a branch point in phyA signal transduction, and locates FHY1 specifically to the proposed cGMP-dependent pathway for phytochrome A FR mediated responses. The proposed Ca$^{2+}$/calmodulin pathway of phytochrome A does not involve FHY1, as the induction of expression of the genes \textit{NR} and \textit{CAB}, which are thought to be regulated by this pathway, is normal in \textit{fhyl}. This contrasts with FHY3 (chapter 4); gene expression in the \textit{fhy3} mutant suggests that FHY3 is specific to the Ca$^{2+}$/calmodulin pathway for phytochrome A signalling, and plays no role in the...
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cGMP-dependent pathway for phyA FR mediated responses. That phyA has a branched signalling pathway, with FHY1 and FHY3 located in separate pathways, is supported by the retained inhibition of hypocotyl elongation under FR observed in *fhyl* and *fhy3*. If phyA signalling is branched then the isolation of mutants displaying a long hypocotyl under FR is not the ideal screen for phyA signal transduction mutants as only *phyA* mutants would display a complete loss of inhibition of hypocotyl elongation. Any signal transduction mutants will retain at least a partial response through a different branch of the phyA signalling pathway. In wild type Arabidopsis the repression of POR expression and turnover of the POR protein (which catalyses a light dependent step in chlorophyll biosynthesis) by prolonged exposure to FR leads to a block in the ability to green under subsequent W or R. Expression of the *PORA* and *PORB* genes, which encode the POR protein for chlorophyll biosynthesis, is not repressed in *fhyl* (or *phyA*) following FR (Barnes *et al.*, 1996b). Therefore, these mutants accumulate POR to the same levels as dark grown seedlings and retain the ability to green under subsequent W or R. This suggests that FHY1 defines a component of the phyA signalling pathway for the FR induced block of greening.

FHY1 is a phyA specific signalling component; however, a role for FHY1 in the regulation of the phyB signalling pathway has now been identified (Cerdán *et al.*, 1999). The phyA signalling pathway regulates the action of the phyB signalling pathway in R (chapter 4); this regulation depends on FHY1.

This information concerning the action of FHY1 provides a good basis for the analysis of suppressors of *fhyl-1*. Mutants isolated can be tested for their suppression of a number of FHY1-mediated responses, such as inhibition of hypocotyl elongation, control of *CHS*, *CHI* expression and greening responses. It is possible that mutants isolated may suppress only some of the *fhyl* phenotypes, these mutants would define branch points in phyA signalling. The isolation and preliminary characterisation of suppressors of *fhyl* is presented in this chapter.
5.2 Results

5.2.1 Screening for suppressors of *fhyl*-1

A screen was developed to isolate suppressors of *fhyl* using seed of *fhyl*-1 mutagenised with different concentrations of EMS (Dr. Praekelt). As described above, *fhyl* displays reduced de-etiolation under FR, the hypocotyl is long and the cotyledons remain closed. The obvious screen for isolation of suppressors of *fhyl* was to grow seedlings under FR and select those seedlings with hypocotyls that were not as long as *fhyl*. Additionally, any seedlings that had long hypocotyls but displayed more cotyledon opening and development than *fhyl* could also be selected as these may suppress a different aspect of the *fhyl* phenotype. There also existed the possibility of isolating mutants that enhanced the *fhyl* phenotype and so might have longer hypocotyls than *fhyl*. Of course these would have to be tested for allelism to phyA.

Mutagenised seed was germinated as described in section 2.1.2. Plates were transferred to continuous FR for up to 48 h and putative mutants selected. EMS 100 (100 mM) lines were screened by Dr. Praekelt. During the duration of this research the EMS 60 (60 mM) lines were screened.

Many potential suppressors of *fhyl* were isolated. These were grown to seed, and screened again under continuous FR.

5.2.2 Optimisation of conditions for FR rescreening

During rescreening of potential *fhyl* suppressors it became apparent that the difference in hypocotyl length between *fhyl* and Laer was not optimal for identification of mutants of intermediate length (figures 5.2, 5.3, 5.4a&c, 5.5b).

Batches of *fhyl* seeds, harvested in different years, were grown next to Laer and phyA under high (~25 μ mol m⁻² s⁻¹) or low (~1 μ mol m⁻² s⁻¹) fluence rates of FR, on different media (Lehle, MS, MS+sucrose(MS30)), and in different FR sources (LEDs or fluorescent tubes), for either 2 or 4 days. The results of this experiment
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suggested that the most pronounced difference in hypocotyl length between Laer and \textit{fhyl} could be observed on Lehle medium, under a low fluence rate of FR (figure 5.1b). Under all conditions the characteristic reduction in de-etiolation of \textit{fhyl} could be observed. However, growth on other media produced a less prominent difference in hypocotyl length (figure 5.1c&d). High fluence rates of FR cause some de-etiolation response in \textit{fhyl} (figure 5.1a). Growing seedlings under LEDs or fluorescent tubes (longer wavelength FR, see appendix b) had no effect on the de-etiolation responses, and de-etiolation was no more apparent at 2 or 4 days. Examples of the effects of the different parameters investigated can be observed in figure 5.1.

For further characterisation of the mutants isolated, seedlings were screened at low fluence rates of FR on Lehle agar for 3d (figures 5.4b and 5.5a).
Figure 5.1 Hypocotyl length of wt, \textit{phyA} and \textit{fhy1} (different years of harvest) under FR fluorescent tubes
a) high fluence rate, Lehle medium
b) low fluence rate, Lehle medium
c) MS30
d) MS

The optimum difference in hypocotyl length between wt and \textit{fhy1} is achieved on Lehle medium under a low fluence rate of FR.
5.2.3 FR screening for *fhy1* suppressors

All putative *fhy1* double mutants were rescreened under continuous FR to confirm altered hypocotyl length or cotyledon expansion. Seedlings were then subjected to a number of monochromatic light treatments. This established whether these mutants were specific to FR or displayed any difference in de-etiolation compared to wt and *fhy1* under other light conditions (figures 5.2-5.5).

5.2.4 R screening of putative *fhy1* suppressors

The *fhy1* mutant does not display any phenotypic alterations under R. Any suppressors of *fhy1* would therefore be expected to have a wt phenotype under R. Mutants that display an alteration in de-etiolation in response to R are unlikely to specifically suppress *fhy1*, these mutants may suppress the phenotype characteristic of *fhy1* by a disruption of a gene with a more global role in control of seedling development. Putative *fhy1* suppressors were screened under continuous R for 3d, examples are presented in figures 5.2-5.5 along with other light treatments.

5.2.5 Phenotype of putative *fhy1* suppressors grown in the dark

True suppressors of *fhy1* would not be expected to display an alteration of dark grown phenotype. Other mutants, such *cop* and *det* may be selected in the screen used for isolation of suppressors of *fhy1*, as these too would be expected to have a short hypocotyl in FR. To eliminate such mutants, seedlings were screened in the dark. In addition, mutants were grown on Lehle medium containing 30 g/l sucrose, as some of the *cop/det* phenotypes are more apparent on such plates. A number of those seedlings selected proved to display a light-grown phenotype in the dark. Examples are presented in figure 5.2.

Mutants numbers 86, 147 and 161 all display increased de-etiolation when compared with *fhy1* under FR. Their de-etiolation under W, R and B is very similar to that of wt, with the exception of 161, which has a shorter hypocotyl under R. All of these mutants have reduced hypocotyl length compared with wt and *fhy1* in the dark, 161 also has expanded cotyledons in the dark. Expansion of cotyledons in dark
is more obvious on plates containing sucrose for 86 and 161. Figure 5.3 shows more mutants isolated which display a short hypocotyl in FR and also have light-grown phenotypes in the dark. Mutant number 2 (figure 5.2a) has opened cotyledons with long petioles in dark. Mutants 64, 67 and 12 all display some characteristics in the dark akin to de-etiolation. Mutant 18 was isolated because of its extremely long hypocotyls under FR, the hypocotyls of this mutant are also long in dark. Although these mutants are potentially interesting they do not form part of the aim of this Ph.D., and as such were excluded from further investigation. The mutants with altered dark phenotypes should be crossed to existing cop/det mutants to check for allelism. Screening for such mutants has been carried out fairly comprehensively, and so it is unlikely that any of those selected define new loci.
Figure 5.2 Putative *sof* mutants which display short hypocotyls in dark
Figure 5.3 Putative sof mutants

a) 64 and 67 display cotyledon expansion and inhibition of hypocotyl elongation in dark
b) 2 and 12 display cotyledon expansion and inhibition of hypocotyl elongation in dark
18 displays long hypocotyls under FR
5.2.6 Selection of mutants for further work

The number of candidates for suppressors of *fhy1* was high so selection of those with the most interesting phenotypes was necessary. Mutants selected showed enhanced de-etiolation responses, compared with *fhy1*, in FR, in at least two different experiments, and on preliminary screening displayed no obvious phenotype under W, R or dark. Figures 5.4 and 5.5 show the preliminary rescreening phenotypes of those mutants initially chosen for further analysis. For ease of identification these mutants were renamed *sof1-sof8*.

Figure 5.4 shows *sof1* and *sof2* under a range of light conditions. These mutants displayed a hypocotyl length in FR which was intermediate between *Laer* and *fhy1*. The *sof1* mutant also displayed greater cotyledon expansion than *fhy1* under FR. The *sof2* hypocotyl was almost as short as that of *Laer*. The phenotype of these mutants is not obviously different from *fhy1* under any of the other light sources, or in the dark. Figure 5.5 shows the remaining mutants selected for further analysis. All of these have reduced hypocotyl length compared with *fhy1* under FR (figure 5.5a). *sof7* and *sof8* also display greater cotyledon expansion under FR (figure 5.5b).

The mutant *sof2* was isolated from the EMS 100 lines, many other potential suppressors of *fhy1* were isolated from these lines. However, the remaining mutants chosen for further analysis were selected from the EMS 60 lines, as the lower concentration of EMS reduced the number of mutations likely to occur in these plants.

Of those mutants selected from EMS 60 for further analysis (*sof1, sof3-8*), some were identified from the same pool of seeds. *sof3* and *sof4* arose in the same pool as each other, and *sof7* and *sof8* arose in the same pool as each other; there was a high chance that these were siblings and so *sof4* and *sof7* were excluded from further experimentation.
Figure 5.4 Phenotype of sof1 (a and b) and sof2 (c) in dark and under a range of light sources.

Under continuous FR sof1 and sof2 display a hypocotyl length intermediate between wt and fhy1, there are no obvious variations from wt under other conditions investigated.
Figure 5.5 Phenotype of sof3-sof8 in dark and under a range of light sources

The sof mutants display hypocotyl lengths intermediate between wt and fhy1 under continuous FR. There are no obvious variations from wt under other conditions investigated.
5.2.7 Backcrossing of mutants to fhyl-1

Those mutants selected for further investigation were backcrossed to fhyl-1. This procedure allows confirmation that the fhyl mutation is still present in these seedlings, and also serves to segregate away some of the additional mutations that may exist within the seedlings selected. There was evidence that these plants contained other mutations as many displayed altered adult phenotypes, for example, large leaves, many meristems, or reduced silique development, when grown for seed. fhyl does not show such characteristics, and so suppressors of fhyl would not be expected to either. Further backcrossing to wt or fhyl is required for elimination of other mutations.

The F1 seed from the sof x fhyl-1 backcross was grown under continuous FR; under such conditions seedlings that are homozygous for fhyl will display a long hypocotyl (assuming that the suppressor phenotypes occur due to recessive mutations). The germination rate of the F1 was poor, and comparison of hypocotyl length was not possible. However, the fhyl mutant does have another characteristic that differs from wt, that is the ability to green following irradiation with prolonged FR. If the putative suppressors of fhyl were wild type for FYH1 then the seedlings would not be expected to green. Suppression of the fhyl phenotype may also lead to a loss of greening ability, however in a backcross to fhyl a recessive sof mutation would not display a phenotype as all seedlings would be heterozygous for the sof mutation. The F1 seedlings were transferred to W following their growth under FR, seedlings greened and were planted out for collection of the F2 seed. So at the F1 stage all sof mutants appeared to still carry the fhyl mutation.

To isolate the homozygous suppressors of fhyl from the F2 population the F2 seeds were screened under FR. Assuming the sof mutation was recessive, 1 in 4 of these seedlings should display the suppressor phenotype. Suppression of the fhyl phenotype may lead, in addition to increased de-etiolation, to a restoration of the “FR induced greening block”. In this case, any sofs would not green following this second round of screening and would be lost. To resolve this problem, the FR was
supplemented with a very low amount of R, this was sufficient to overcome the FR greening block but not to cause complete de-etiolation of the seedlings.

Seedlings that displayed a shorter hypocotyl were grown for F3 seed. These shorter hypocotyl phenotypes occurred in a ratio of approximately 1:3 short:long, suggesting that all of the sof mutants arose due to recessive mutations. Seedlings were labelled with a number corresponding to the F2 batch, and a letter denoting the particular seedling within that batch, for example sof 1-A, was isolated from the sof x fhy l, F2 plant number 1, and was the first seedling (A) from the second round screening for a short hypocotyl in FR.

These mutants contain the fhy l mutation and have been isolated by their increased de-etiolation compared with fhy l.

5.2.8 Hypocotyl length of backcrossed sof s under FR
Following the backcross and selection of sof s, confirmation of their short hypocotyl phenotype was determined by growth under FR (figure 5.6). Of the sof mutants selected as short in FR, sof 1-D is fhy l, this seedling probably had a slower germination rate. sof 1-A, 1-B and 1-C all display a hypocotyl length intermediate between Laer and fhy l, as expected of a partial suppressor of fhy l (figure 5.6). sof 1-E has hypocotyls of the same length as wt; this could suggest that the sof mutation is semi-dominant, with sof 1-E being homozygous, and the others heterozygous. Alternatively sof 1-E could be a wild type contaminant. This could be resolved by backcrossing of sof 1-E again to fhy l, if this batch is wt then all of the F1 will be short in FR. Alternatively, the next generation of the other sof batches could be screened for segregation of hypocotyl length. With knowledge of the genetic lesion in FHY1 it may be possible to design primers for PCR identification of seedlings containing the fhy l mutation.

All of the remaining sof fhy l-l mutants selected displayed a hypocotyl length intermediate between that of wt and fhy l.
Figure 5.6 Hypocotyl phenotype, under FR, of sof mutants backcrossed to fhy1

All sof mutants display hypocotyl elongation less than that of fhy1 but greater than that of wt. The loss of inhibition of hypocotyl elongation in fhy1 is suppressed in these mutants.
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Figure 5.7 Average hypocotyl length of \textit{sof} mutants in dark and FR

All \textit{sof} mutants display hypocotyl elongation less than that of \textit{fhy1} but greater than that of wt. The loss of inhibition of hypocotyl elongation in \textit{fhy1} is suppressed in these mutants.

Error bars represent value for 95\% confidence interval. Approximately 30 seedlings were measured for each point.
Seedlings were grown under FR to determine the severity of these sof \textit{fhy1-1} mutations. It is clear that the hypocotyl lengths of the sof \textit{fhy1-1}s is intermediate between those of \textit{fhy1} and wt, strongly suggesting that these mutants partially suppress the \textit{fhy1} phenotype (figure 5.7). The sof2 mutant has a much reduced hypocotyl length compared with that of \textit{fhy1}, and as such displays the strongest suppression of the \textit{fhy1} phenotype. sof3, sof5 and sof8 are comparatively weak suppressors of \textit{fhy1}.

\textbf{5.2.9 Hypocotyl length of backcrossed sofs under R and B}

The backcrossed sof mutants were rescreened in R to check for any altered de-etiolation phenotypes which might not have been observed before backcrossing, if for example other mutations were present. Figure 5.8 shows the hypocotyl length of the sof\textit{fhy1-1} mutants compared with wt and \textit{fhy1} under R. The sof2 mutant appears to have shorter hypocotyls than wt or \textit{fhy1}. This could indicate that the sof2 mutation causes a more pleitropic phenotype, affecting hypocotyl inhibition under a number of conditions. However, it is possible that the action of SOF2 in wt is to exert a negative control of hypocotyl elongation; in the \textit{fhy1} background in FR the loss of SOF2 would lead to inhibition of hypocotyl elongation. In R the loss of SOF2 would also lead to greater hypocotyl inhibition and therefore shorter hypocotyls. The other sof \textit{fhy1-1} mutants display similar inhibition of hypocotyl elongation to wt and \textit{fhy1}, suggesting that phyB responses to R are not suppressed by the sof mutations.
Figure 5.8  *sof fhy1-l* mutants grown under R

*sof2* appears to have shorter hypocotyls than wt or *fhy1*. Other *sof* mutants do not display any obvious altered phenotypes in R.

Figure 5.9  *sof fhy1-l* mutants grown under B

*sof2* and *sof3* have slightly shorter hypocotyls than wt or *fhy1* in B. Other *sof* mutants do not display any obvious altered phenotypes in B.
The hypocotyl length of \( fhy1 \) under B is slightly longer than that of wt (Whitelam et al, 1993). This may occur due to the small action of phyA in B. The \( sof fhy1-1 \) mutants were screened under B to determine whether this \( fhy1 \) phenotype was suppressed (figure 5.9). Under the conditions used no effect of the \( fhy1 \) mutation upon inhibition of hypocotyl elongation was observed, however, seedlings were much shorter than those shown in the paper describing \( fhy1 \) (Whitelam et al, 1993) due to different fluence rates and light sources. The \( sof2 \) and \( sof3 \) mutants may be slightly shorter than wt, but the remaining \( sof fhy1-1 \) mutants are of a similar length to wt and \( fhy1 \). The mutant phenotype of \( sof1, sof5 \) and \( sof8 \) seems to be specific to FR induced de-etiolation, whereas \( sof2 \) and \( sof3 \) show increased inhibition of hypocotyl elongation under other monochromatic light sources.

### 5.2.10 Greening of \( sof \) mutants

As described earlier, wt Arabidopsis displays a FR block on greening, seedlings grown in FR are unable to produce chlorophyll on transfer to W. The \( fhy1 \) mutation allows seedlings to green on transfer from FR to W. Suppression of \( fhy1 \) may occur only in the restoration of de-etiolation, i.e. affecting only a subset of the responses affected by FHY1. Alternatively, more or all of the \( fhy1 \) phenotypes may be suppressed, in which case the \( sof \) mutants would not be expected to green in W following pre-irradiation with FR. This possibility was tested. Laer, \( fhy1 \) and the \( sof fhy1-1 \) mutants were grown for three days in FR and transferred to W for four days. All Laer seedlings failed to green, whereas all \( fhy1 \) and \( sof fhy1-1 \) mutants displayed 100% greening. This suggests that only some \( fhy1 \) phenotypes are suppressed in the \( sof \) mutants.

### 5.2.11 Anthocyanin production

FHY1 is thought to play a role in the putative cGMP pathway which mediates CHS production. The \( fhy1 \) mutant has reduced expression of CHS under FR (Barnes et al, 1996). One indirect indicator of the amount of CHS is the accumulation of anthocyanin within seedlings. Wt Arabidopsis accumulates anthocyanin in FR, whereas \( fhy1 \) has a very reduced level. The amount of anthocyanin accumulated in
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the sof fhy1-1 mutants was measured after three days in FR (section 2.1.8). The results of this experiment are presented in figure 5.10. Laer clearly accumulates anthocyanin, whereas fhy1 accumulates very little. The amount of anthocyanin accumulated in the sof fhy1-1 mutants is intermediate between Laer and fhy1, providing further evidence for the partial suppression of fhy1.

The sof1 1-E batch does not display wt levels of anthocyanin accumulation suggesting that it is not a wt contaminant, however, it does not display higher levels of anthocyanin than sof1 1-A which would be expected if it were homozygous and sof1 1-A heterozygous.

Comparison of the inhibition of hypocotyl elongation and the anthocyanin accumulation under FR (figures 5.8 and 5.10) demonstrates a correlation between the suppression of these two characteristics. The sof1 and sof2 mutants which display greatest suppression of fhy1 for inhibition of hypocotyl elongation also show the greatest accumulation of anthocyanin. This suggests that these mutants have a greater suppression of the fhy1 phenotype than the other sof mutants.

5.2.12 Hypocotyl elongation in photoperiods

The hypocotyl of fhy1 under continuous W irradiation is of the same length as that of wt, however under light/dark cycles (8h light/16h dark) the hypocotyls of fhy1 are more elongated than those of wt (Johnson et al, 1994). The sof fhy1-1 mutants were grown under short day conditions for seven days, figure 5.11 shows these seedlings. The fhy1 seedlings are slightly longer than those of wt, the sof fhy1-1 mutants appear to have shorter hypocotyls than those of fhy1. The hypocotyls of sof1 1-E are shorter than the wt seedlings. This suggests that sof1 1-E may be more than suppressing the fhy1 phenotype, and may suggest that in sof1 1-E the normal FHY1 responses are amplified.

More detailed analysis of this phenotype for all sof mutants should be performed for further characterisation.
Figure 5.10 Anthocyanin accumulation in sof mutants following 3d FR. 50 seedlings were used for each bar. Repetition of this experiment with more replicates is required for confirmation of these results.

Anthocyanin accumulation in Laer is much higher than that of fhyl, all fhyl sof double mutants accumulate anthocyanin to levels intermediate between these, suggesting a partial suppression of this fhyl phenotype.
Figure 5.11 Seedlings grown under short days (8h light/16h dark) for 7 days
In this preliminary screen $fhyl$ is slightly longer than wt under SD, the sof mutants are shorter than $fhyl$ suggesting suppression of this SD response.
Figure 5.12  sof mutants grown under long days (12h light/12h dark)
There is no obvious alteration of adult phenotype for sof mutants grown under LD.
5.2.13 Adult phenotype of sof mutants

The adult phenotype of fhyl is not obviously different from that of wt. However, the sof fhyl-1 mutants were grown in LD and SD for observation. Before backcrossing many unusual adult phenotypes had been observed in the sof mutants, such as large rosette leaves or reduced apical dominance. The backcrossed sof were observed for the presence of any characteristics which might indicate a more global effect of the mutation on plant development. No obvious changes in morphology were observed in the majority of the sof fhyl-1 mutants compared with wt and fhyl under LD (12h light/12h dark) (figure 5.12).

5.3 Discussion

The mutagenesis of fhyl-1 seeds has enabled screening for and isolation of suppressors of fhyl. The identification of a number of suppressors of fhyl is presented here, with their preliminary characterisation. The mutants isolated display a greater inhibition of hypocotyl elongation under FR compared with that of fhyl, but this inhibition is less than that occurring in wt. The mutants accumulate anthocyanin to levels between those of Laer and fhyl, and the amount of anthocyanin accumulation corresponds with the degree of suppression of the fhyl phenotype in terms of inhibition of hypocotyl elongation. The sof mutation may be semi-dominant (section 5.2.8), whereas the remaining mutations appear to be recessive. sof1, sof5 and sof8 display enhanced inhibition of hypocotyl elongation only under FR, whereas sof2 and sof3 display shorter hypocotyls in R and/or B. Backcrossing of these mutants to fhyl-1 demonstrated that the fhyl-1 mutation was still present, as segregation of long and short hypocotyls was observed in the F2. The ability of all sof fhyl-1 mutants to green following transfer from FR to W also supports the existence of the fhyl mutation, and demonstrates that this aspect of the fhyl phenotype was not suppressed by the sof fhyl-1 mutations. These mutants potentially define previously unknown components of the phyA signal transduction pathway, and further characterisation may increase understanding of this intricate pathway.
The sof fhyl-1 mutants isolated do not display an aberrant phenotype in the dark and therefore the mutant phenotype is light-dependent. The partial suppression of the fhyl phenotype in FR suggests that these mutants define components of the phyA signalling pathway. The action/role of SOF(s) could occur via a number of mechanisms to affect the responses mediated by phyA. FHY1 is a positive regulator of phyA signalling in FR, demonstrated by the reduced inhibition of hypocotyl elongation in FR. However, some phyA action still occurs in fhyl, and the levels of phyA apoprotein are normal. The SOF protein(s) may act to negatively regulate the action of FHY1 through the FR HIR. In the fhyl background the reduction of this negative regulation, such as in the sof mutants, would lead to increased action through the HIR, mediating greater inhibition of hypocotyl elongation. Alternatively, the SOF loci may lie in pathways distinct from those involving FHY1, the action of these pathways could be negative regulation of hypocotyl inhibition. If this was the case, in wt plants the hypocotyl length in FR would result from a compromise between this pathway and that involving FHY1, to mediate inhibition of hypocotyl elongation. Mutations occurring at the SOF loci would allow greater action through the FHY1 pathway, leading to increased inhibition of hypocotyl elongation. Of course in the fhyl background this inhibition would not be maximal and may manifest as an intermediate hypocotyl length. If the action of SOF(s) in FR is the negative regulation of FHY1, or to directly prevent hypocotyl inhibition, then the sof mutations in a wt background would be expected to cause a greater inhibition of hypocotyl elongation than observed in wt. This could be tested by analysis of sof mutants crossed to wt to remove the fhyl mutation.

FHY1 and FHY3 are known to affect different branches of phyA signalling, both mediating inhibition of hypocotyl elongation, and it is therefore possible that SOF(s) act as negative regulators of FHY3. In the sof mutants greater inhibition of hypocotyl elongation could occur due to increased FHY3 action. It would be interesting to cross the sof mutations into the fhyl3 background, and also into the phyA background to observe if the phenotypes associated with these mutations are also suppressed. Anthocyanin levels in the sof mutants, correlate with their
inhibition of hypocotyl elongation. This suggests that it is action through the FHY1 cGMP pathway that is affected by the sof mutations, and does not fit with the theory of SOF action on FHY3 which may play a role only in the Ca\(^{2+}\)/calmodulin pathway (see chapter 4).

Greater inhibition of hypocotyl elongation, and greater accumulation of anthocyanin could arise in the sof mutants if the level of phyA protein was increased. It is important to ascertain whether these mutants have higher levels of phyA than wt, or fhyl seedlings. Overexpression of phyA in transgenic seedlings increases sensitivity to FR and to R (Boylan and Quail, 1992). The suppressors of fhyl do not display greater inhibition of hypocotyl elongation under R, so this aspect of phyA overexpression is not apparent suggesting that the levels of phyA may be normal, or at least only very slightly elevated in the sof mutants.

If the sof mutants are not allelic and define different components of the phyA signal transduction it may be possible that all of the above scenarios for inhibition of hypocotyl elongation in the fhyl background could exist. The fact that the fhyl phenotype is only partially suppressed in the mutants isolated could suggest that these mutants have only a partial loss of function, or that more than one gene is responsible for this function. Expression analysis of the SOF gene(s) within the sof mutants will address this problem.

In summary, the regulation of inhibition of hypocotyl elongation by phyA under continuous FR may involve a branched pathway- with fhyl and fhys defining two of these branches. This would hypothesis fits with the retained inhibition of hypocotyl elongation under FR of both fhyl and fhys, and by the different induction of gene expression in these two mutants. Therefore, the sof may not be genuine suppressors of fhyl, but could regulate other branches of the phyA signalling pathway, for example that defined by fhys. The increase in anthocyanin accumulation in the sof compared with that in fhyl provides circumstantial evidence that the sof are suppressors of fhyl as CHS regulation is unaffected in fhys (chapter 4).
5.4 Proposal of future sof analysis

The analysis of the sof mutants is at a very early stage, and there is much scope for future work to determine the role of SOF in the phyA signalling pathway. The sof mutants should be crossed with wt for the selection of monogenic sof mutations. These mutants could then be screened for any phenotypes in the presence of FHY1, which may indicate a broader role of these loci than involvement with the FHY1 branch of the phyA pathway. Another important step will be to cross these mutants to each other to check for allelism; in the isolation of spa1 (Hoecker et al, 1998) five mutants were found to suppress the phyA phenotype and these were allelic. The difference in the degree of fhy1 suppression may be accounted for by mutation of different genetic loci or may be simply due to different levels of gene expression caused by different mutations of the same gene. Of course the sof mutants should also be checked for allelism with spa1 which was isolated by its short hypocotyl phenotype in FR. It is possible that the sof mutations are intragenic, to determine whether the sof mutations are intragenic or extragenic crosses to wt could be performed, and the ratio of wt to mutant phenotype scored. It is also possible that the sof mutation is allele specific, i.e. a second mutation within the fhy1-1 gene restoring normal activity. In this case the sof phenotype would only be observed in fhy1-1, crosses to other fhy1 alleles could be performed. If suppression of the fhy1 phenotype occurs in other alleles then the mutation is not specific to fhy1-1.

Analysis of anthocyanin accumulation suggests that the induction of the CHS and CHI genes is at least partially restored in the sof mutants. Northern blotting should be used to confirm induction of these genes. Screening at different time points will confirm whether or not the kinetics of this induction differ from wt. Additionally, the levels of PC and RBCS expression should be investigated; it would be expected that a suppressor of fhy1 restored expression of these genes. However, it is possible that not all of the fhy1 phenotypes are suppressed; SOF may play a role in only some phyA/FHY1 responses, and may therefore only restore some fhy1 phenotypes to wt. It would be interesting to investigate the levels of expression of these genes in both the monogenic suppressors and the sof fhy1 double mutants. The monogenic sof
mutations may lead to amplified expression of these genes, or alternatively the action of SOF may only be apparent in the absence of FHY1.

The sof mutations do not restore the wt FR greening block, possibly due to only the partial suppression of the fhyl phenotype (the exception being sof1-1-E which does display some block of greening, but this may be wt). The partial suppression of fhyl phenotype for inhibition of hypocotyl elongation, but retained greening ability in the sof mutants provides circumstantial evidence for another branch of the phyA signalling pathway. POR could be at the end of a specific branch of the phyA signalling pathway which occurs after FHY1 but is not affected by SOF. The presence or absence of the FR greening block in monogenic sofs may provide interesting information concerning the action of SOF. If the monogenic sof mutants display a wt FR greening block then the action of SOF is only apparent in the absence of FHY1. However, if like fhyl, the sof mutants have the ability to green then these mutants define components of the phyA pathway mediating the FR block of greening.

Other observations of altered phenotype have been observed in fhyl, the restoration of these characteristics to wt could also be investigated. For example, fhyl seedlings display greater hypocotyl elongation than wt under low R/FR ratios, and under extended SD conditions have three more leaves at flowering than wt (Johnson et al, 1994).

Identification of the FHY1 gene has not yet been published, details concerning FHY1 and the molecular lesion in fhyl-1 will greatly assist in future work. When more knowledge concerning the role of FHY1 is revealed it will be possible to speculate on the function of SOF(s) in more detail. Future work on the sof mutants should include mapping of these mutations, to confirm that they define novel loci. Cloning of these genes should then proceed to identify the structure of the proteins produced, for speculation on their cellular role, and location. Expression studies,
such as those described for *GIL1* in chapter 3, will provide more information concerning the role of the SOF protein(s).
6.0 General Discussion

The isolation of photomorphogenic mutants of Arabidopsis has, over recent years, greatly increased understanding of the roles of phytochromes, and how perception of light induces changes in plant morphology. As elucidation of phytochrome signal transduction pathways progresses the complex nature of these pathways becomes apparent. This thesis describes further characterisation of the previously known fhy3 mutant, and also presents new mutants that define points of interaction between light perception and gravity perception (gill), and new components of the phyA signal transduction pathway (sofs). Through analysis of these mutants important information concerning signalling interaction has arisen.

The phytochrome A pathway is perhaps the best characterised of the phytochrome signalling pathways, through analysis of mutants and overexpression studies. The mutants of fhy1 and fhy3 (Whitelam et al, 1993) were the first that defined components of a phytochrome signalling pathway, with FHY1 and FHY3 being specific to the phyA pathway. In more recent years other mutants defining signalling components have been isolated, some specific to phyA, such as far1 (Hudson et al, 1999), spal (Hoecker et al, 1998), and some specific to phyB, e.g. redl (Wagner et al, 1997). Other mutants such as psi2 (Genoud et al, 1998) display aberrant phenotypes in FR and R and are thought to define components for regulation of both the phyA and the phyB pathways. This provides supporting evidence that there is some interaction between the phyA and phyB pathways, either sharing some of the same components, or by crosstalk with the action of one pathway affecting the action of the other.

There is mounting evidence for crosstalk between the phyA and phyB pathways. Under R, the predominant phytochrome action is that of phyB, however phyA also plays a role in partial de-etiolation under R and exerts a negative effect on the LFR mediated by phyB (Mazzella et al, 1997). The phyB response to a pulse of R can be amplified by pre-irradiation with FR perceived by phyA. So phyA and phyB interact
in both an antagonistic and synergistic manner. It would not, therefore, be
unexpected to find shared components of these pathways, or components of one
pathway which act to regulate the other. This research shows that the \textit{fhy3} mutant
defines such a component. The role of FHY3 in FR is to mediate some inhibition of
hypocotyl elongation. Under R FHY3 plays a role in negative regulation of the phyB
pathway for inhibition of hypocotyl elongation and for cotyledon expansion (chapter
4). In R some \textit{fhy3} alleles have greater inhibition of hypocotyl elongation and
greater cotyledon expansion than wt. This phenotype could be due to either
enhanced phyA action in R, or enhanced phyB action. The interaction of FHY3 and
the phyB pathway is demonstrated by the analysis of the \textit{fhy3 phyB} double mutant,
suggesting that the \textit{fhy3} phenotype in R is due to phyB action rather than phyA
(chapter 4). This suggests that FHY3 plays a role in regulation of both the phyA and
phyB signalling pathways in some way. The \textit{sof2} mutant (chapter 5) also potentially
defines a component of regulation of both the phyA and phyB pathways, displaying
greater inhibition of hypocotyl elongation under FR than \textit{fhyl}, and under R
displaying greater inhibition of hypocotyl elongation than wt and \textit{fhyl}. Further
analysis of this mutant may advance understanding of phyA and phyB interaction.

The response modes (VLFR, LFR and HIR) of phytochrome are genetically
separable, demonstrated by the identification of the \textit{VLF} loci (Yanovsky \textit{et al,}
1997). The work presented in this thesis provides supporting evidence for genetic
separation of the response modes. The \textit{fhy3} mutant retains some phyA responses in
FR as some inhibition of hypocotyl elongation is evident in this mutant (figure 4.2-
4.4). The VLFR of \textit{fhy3} is retained; it is only at high fluence rates of continuous FR
that hypocotyl length in \textit{fhy3} is obviously greater than that of wt (figure 4.2-4.4). In
fact \textit{fhy3} does not lack the HIR but this response is shifted significantly to higher
fluence rates (Yanovsky pers. com.). For the VLFR of \textit{fhy3} to be normal and for the
HIR to be altered there must be some separation of these two response modes. In
contrast the \textit{fhyl} mutant displays a normal HIR but a reduced VLFR, again
supporting separation of the two response modes. The isolation of mutants which
suppress the \textit{fhyl} phenotype under FR, as presented in this thesis (chapter 5), may
identify components of the phyA pathway which also support separation of the response modes.

Signalling by phytochromes has been proposed to occur through two pathways, that of cGMP and that of Ca\(^{2+}\)/calmodulin (Bowler et al, 1994). FHY1 may act through the proposed cGMP pathway (Barnes et al, 1996), as demonstrated by the reduced induction in \(fhy1-1\), under FR, of genes such as \(CHS\), \(CHI\), \(RBCS\) and \(NR\), which appear to be regulated by this pathway. The expression of \(CAB\) in \(fhy1-1\) is unaffected - this gene is thought to be regulated by the Ca\(^{2+}\)/calmodulin pathway. In contrast, as the gene expression data presented in chapter 4 suggests, FHY3 may act through the Ca\(^{2+}\)/calmodulin pathway. Expression of \(CAB\) in the \(fhy3\) mutant is altered, whereas expression of other genes such as \(CHS\) and \(CHI\) is unaffected. However, RBCS expression under FR may be reduced in \(fhy3\). So, although the \(fhy1\) and \(fhy3\) mutants both display long hypocotyls under FR they may define components of separate branches of the phyA signalling pathway. If inhibition of hypocotyl elongation in FR is regulated by more than one branch of the phyA signalling pathway screening for further signal transduction mutants by hypocotyl length may be inappropriate. Action through the branches of the phyA signalling pathway mediates expression of different genes, and one of these pathways operates the VLFR while another operates the HIR.

Mutants that suppress the \(fhy1\) long hypocotyl phenotype may define components of pathways in which FHY1 is predicted to act, for example the cGMP pathway, and in terms of responses modes, the VLFR. The SOF proteins may act in these pathways alone defining components of only these branches. However, as described above, the same hypocotyl phenotype may be produced by mutation of different pathway branches. So it is equally possible that the \(sof\) mutant phenotypes arise by increased action through other pathways, such as those involving FHY3, as this may ultimately result in the inhibition of hypocotyl elongation under FR.
The *SPA1* gene encodes a novel protein with two nuclear localisation signals and putative coiled-coil domains which promote protein-protein interactions (Hoecker *et al*, 1999). The SPA1-GUS fusion was found almost exclusively in the nucleus. So the SPA1 protein could directly regulate gene expression, or may regulate the activity of phyA or a phyA signalling intermediate. The identification of PIF3 (Ni *et al*, 1999) which interacts with both phyA and phyB in the nucleus provides support to the suggestion of a nuclear role for SPA1 in regulation of gene expression. The *sof* mutants described in this thesis may define components which act in a similar manner to SPA1. SPA1 and SOF(s) could form part of the same pathway for regulation of phyA, or could form a complex for direct interaction with phyA in the nucleus. Of course this is speculative, and detailed molecular analysis of the *sof* mutants is required before any particular SOF action can be assigned. It will be interesting to determine whether the SOF proteins play a role analogous to that of SPA1 or whether they act via a different mechanism to regulate phyA signalling in FR. The regulation of SPA1 by phyB, and the interaction of phyA and phyB pathways demonstrated by analysis of *fhy1* and *fhy3*, suggest that many interactions or points of crosstalk may exist between phyA and phyB signalling. The activity of the SOF(s) in R, and regulation of activity in R by phyA or phyB will be important for determination of their roles in phytochrome signalling.

The interaction of phytochrome signalling and gravity perception in Arabidopsis has been characterised by the analysis of *phyA* and *phyB* mutants (Poppe *et al*, 1996, Robson and Smith, 1996). The randomisation of hypocotyls under R occurs due to action of both the phyA and phyB pathway. Either the phyA or the phyB pathway is sufficient to mediate randomisation of hypocotyls, demonstrated by the retention of this response in *phyA* or *phyB* mutants (Poppe *et al*, 1996, Robson and Smith, 1996). Hypocotyl randomisation is not mediated by any other phytochromes, as the *phyAphyB* double mutant is gravitropic (Robson and Smith, 1996). The *gilI* mutant (chapter 3) is gravitropic under R and pulses of FR, however, perception of gravity is not completely disabled in this mutant as some randomisation can be observed under continuous FR. By comparison with *phyA*, *phyB* and *phyAphyB* mutant
phenotypes it can be proposed that GIL1 plays a role in both phyA and phyB mediated agravitropism. Therefore, GIL1 may define a shared component of the phyA and phyB signalling pathways for responses to gravity, or may define a point at which the pathways merge for mediation of agravitropism.

Analysis of the gene, K19M22.14, thought to encode GIL1 yields no information for a role of GIL1 within the cell. There are no nuclear localisation signals in K19M22.14, which suggests that although phyA and phyB have recently been identified in the nucleus, this is not the place of interaction with K19M22.14. K19M22.14 does not contain any recognised domains for protein interactions or DNA binding activity, and so how phyA and phyB signalling is affected by this protein is difficult to predict. Future analysis to determine the distribution of GIL1 both within the cell and the whole plant may yield more information to address this question.

The role of phytochrome mediated agravitropism is unresolved. It can be speculated that in light phyA and phyB act to disable gravitropism, and so prevent gravitropic compensation, this would maximise the seedlings response to a phototropic stimulus. Under monochromatic R or FR this phototropic stimulus is not perceived and the result is randomisation of the hypocotyls. So GIL1 may play a crucial role in this "switching off" of gravitropic responses in light to enable seedling orientation in an appropriate direction.

In conclusion the research presented in this thesis strongly supports the interaction of phytochrome pathways with each other and with other signalling pathways such as that of gravity. The fhy3 mutant provides evidence for interaction between phyA and phyB signalling pathways for mediation of de-etiolation; the gill mutant provides evidence for the interaction between phyA and phyB signalling pathways and responses to gravity perception. In addition new mutants (sofs) are described which may yield more information concerning the regulation of phyA signalling under FR, and possibly under R.
Appendix 1

Primers

Primers for Genome Walking:
AP1: GTA ATA CGA CTC ACT ATA GGG C
AP2: ACT ATA GGG CAC GCG TGG T
BAR1: CAA CCC TCA ACT GGA AAC GGG CCG GGA
BAR2: CGT GTG CCA GGT GCC CAC GGA ATA GT

Primers for confirmation of T-DNA insert and genes near to insert position:
all primers designed with 50% AT, 50% GC. PCR cycles: 94°C 45 sec, 55°C 1 min, 72°C 30 sec, 30 cycles.

Primer pair 1: small region between K19M22.13 and K19M22.14, close to proposed T-DNA insertion site. Product = 570bp.

5-1: GAA GAT GTT GCC AGG CAT AC
3-1: CTT ATT GCA CAC ATG GCT GT


5-2: AGA GAG GAG AAA TGG CGA AC
3-2: ACC AAT CCT TCT CAG CTC AG


5-3: CCA TGA TAT AGC CGG CTG TA
3-3: GGC ATC ATC ATC GGA TCC TA
Appendix 1

Primer pair 4: small region of K19M22.12 (homology to receptor protein kinase).
Product = 590bp.

5-4: CCT TGC TCG TTT AGT GTC GG
3-4: TCT TCA ATC CTC GGC AAT GC

Primer pair 5: small region of K19M22.15 (mitochondrial uncoupling protein).
Product = 510bp.

5-5: AGC AAT CGA CTG TGT TTC GG
3-5: CGT CTC ATC ATG TGT GGT AG

Primers for 3' and 5'RACE
SMART II oligonucleotide: AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG

NUP: AAG CAG TGG TAA CAA CGC AGA GTA C

Universal primer mix:
0.2 µM Long universal primer (LUP):
CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACA ACG CAG AGT
1 µM Short universal primer (SUP):
CTA ATA CGA CTC ACT ATA GGG C

3' RACE cDNA synthesis primer: AAG CAG TGG CAA CGC AGA GTA (T)₁₇ N₁₇ N

5’ RACE cDNA synthesis primer: (T)₁₇ N₁₇ N
(N=A, C, G or T; N₁₇=A, G or C)

GU3-8: CCG CCT CAT AAG GCG CCA CGG CC

GU5-8: CCA CTC TCC GCC GGC CGG CAA G
Primers for amplification of *GIL* for complementation of *gilI*

5RACEgil: CAT CGC CAT ATC AGC ATC GTG CAT C

EcoRI5-1: CCG AAT TCG AAC ATG TGT ACC TAG CTA GC

Xba3-6: GGT CTA GAA TGA TGG GAG ACT CTA C
Appendix 2

Monochromatic Light Source Scans

Figure A1 Scans of red light sources
Figure A2 Scans of far-red light sources
Literature cited


Literature cited


Literature cited


