A Genetic Approach to Understanding the Responses of *Arabidopsis thaliana* to Ultraviolet-B Light

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To my parents, Robert and May Brown

Who have provided me with every support and encouragement

I have yet to see any problem, however complicated, which when you looked at it in the right way, did not become still more complicated.

POUL ANDERSON

Taken from Barrow, J. D. (1992). Theories of Everything. London, Vintage.

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ABBREVIATIONS

ACE	ACGT-containing element
ATase2	glutamine phosphoribosyl pyrophosphate amidotransferase (isoenzyme 2)
atd2	ATase2 deficient mutant
bc2	backcrossed twice to wild-type (suffix)
bHLH	basic helix-loop-helix
bZIP	basic leucine zipper
C4H	cinnamate-4-hydroxylase
CAB	LHCB1 chlorophyll a/b binding protein
CHI	chalcone isomerase
CHR	chalcone reductase
CHS	chalcone synthase
chom	chalcone synthase overexpressing mutant
chum	chalcone synthase underexpressing mutant
cia1	chloroplast import apparatus deficient mutant
CKII	casein kinase II
4CL	4-coumaroyl:CoA-ligase
Col	Columbia
COP	constitutive photomorphogenic
CPDs	cyclobutane pyrimidine dimers
CRY	cryptochrome
CSN	COP9 signalosome
cue	chlorophyll a/b binding protein (CAB) underexpressing mutant
DEPC	diethyl pyrocarbonate
DET	de-etiolated
DFR	dihydroflavonol 4-reductase
DMID	7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase

DMSO	dimethyl sulfoxide
DOV1	differential development of vascular associated cells
ds	double stranded
EDTA	diaminoethanetetra-acetic acid
ELIP	early light-induced protein
EMS	ethyl methane sulfonate
EtBr	ethidium bromide
EtOH	ethanol
F_1	first filial generation
F_2	second filial generation
FAD	flavin adenine dinucleotide
fah1	ferrulate-5-hydroxylase deficient mutant
F3H	flavanone 3-hydroxylase
FMN	flavin mononucleotide
FR	far-red light
FSI and FSII	flavone synthase
F3'H	flavonoid 3' hydroxylase
F3'5'H	flavonoid 3'5' hydroxylase
GeTCID	gene transfer clone identification and distribution service
GUS	β-glucuronidase
H_2O_2	hydrogen peroxide
HFR	high fluence response
HW	high white light
HY	long hypocotyl
ICX	increased chalcone synthase expression
IOMT	isoflavone *O*-methyltransferase
IFR	isoflavone reductase
I2'H	isoflavone 2'-hydroxylase

IFS	isoflavone synthase
JA	jasmonic acid
LCR	leucoanthocyanidin reductase
LDOX	leucoanthocyanidin dioxygenase
L. *er*	Landsberg *erecta*
LFR	low fluence response
LOV	light / oxygen / voltage
LRU	light responsive unit
Luc	luciferase
LW	low white light
M_1	plant grown directly from mutagenized seed
M_2	second generation mutagenized plant
MATE	multidrug and toxic compound extrusion
MRE	MYB recognition element
NASA	National Aeronautics and Space Administration
NASC	Nottingham *Arabidopsis* Stock Centre
NCBI	National Center for Biotechnology Information
NER	nucleotide excision repair
NO	nitric oxide
NOS	nitric oxide synthase
NPH	non-phototropic hypocotyl
NPL	non-phototropic hypocotyl-like
OAc	acetate
OMT	*O*-methyltransferase
6-4 PPs	pyrimidine (6-4) pyrimidone photoproducts
PAL	phenylalanine ammonia-lyase
PAS	Per / Arnt / Sim
PEP	phosphoenolpyruvate

P_{FR}	far-red light absorbing form of phytochrome
pH	$-\log_{10}$ (hydrogen ion concentration)
PHOT	phototropin
PHR1	*Arabidopsis* type II CPD photolyase
PHY	phytochrome
PR	pathogenesis-related
P_R	red light absorbing form of phytochrome
psi	pounds per square inch
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RBCS	ribulose-1,5-bisphosphate carboxylase small subunit
RCC	regulator of chromatin condensation
ROS	reactive oxygen species
35S	cauliflower mosaic virus 35S promoter
SA	salicylic acid
ss	single stranded
STS	stilbene synthase
TAIR	The *Arabidopsis* Information Resource
T-DNA	Transfer DNA
tt	transparent testa
UFGT	UDPG-flavonoid glucosyl transferase
uli3	UV-B light insensitive mutant
USDA	United States Department of Agriculture
UV	ultraviolet
UVR	UV resistance locus
VLFR	very low fluence response
VR	vestitone reductase
v/v	volume / volume
WT	wild type

w/v	weight / volume
X-Gluc	5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronide

SUMMARY

A decrease in stratospheric ozone over the past few decades has resulted in an increase in the quantity of UV-B (280-320 nm) reaching the surface of the Earth. Paradoxically, plants can both suffer from UV-B induced damage and benefit from utilizing UV-B as a trigger for developmental responses and gene expression. There is almost certainly more than one pathway by which plants transmit a signal after perceiving UV-B, in order to effect appropriate responses. Although it is not known how serious the implications of increased ambient UV-B will be for the yield of important crops in the future, our lack of understanding of the mechanisms by which UV-B elicits different responses from plants depending on fluence rate, wavelength and developmental stage ought to be redressed.

In order to try to identify some of the proteinaceous components involved in UV-B perception and signal transduction in *Arabidopsis* we adopted a genetic approach. Various responses to UV-B were carefully investigated with a view to identifying those most suitable to form the bases for genetic screens. It was demonstrated that UV-B wavelengths are capable of stimulating positive phototropic hypocotyl curvature even in the absence of the phototropins (phot1 and phot2), although a screen for mutants lacking this response to UV-B proved unsuccessful.

By contrast, a screen for mutants altered in UV-B induced *CHALCONE SYNTHASE* (*CHS*) gene expression facilitated by a *CHS* promoter – *Luciferase* (*Luc*) reporter gene, produced four under-expressing and two over-expressing mutants. The four under-expressing mutants were each shown to have single base pair changes in the *UV RESISTANCE LOCUS 8* (*UVR8*) gene. An *Arabidopsis uvr8* mutant had previously been isolated by Kliebenstein *et al.* in a screen for plants exhibiting hypersensitivity to supplementary UV-B induced damage. Reduced levels of *CHS* transcript accumulation in response to UV-B had also been previously observed in the mutant. However, characterization of *uvr8* mutants was substantially extended in the work described here. The involvement of UVR8 in a UV-B specific branch of

photoreception / signal transduction is strongly supported by the *uvr8* mutants' retention of *CHS* expression in response to UV-A and cold treatment in mature plants and to far-red and sucrose treatment in seedlings. UVR8 was shown to be necessary for UV-B (but not UV-A) induced transcript accumulation of the LONG HYPOCOTYL 5 (HY5) transcription factor, and the expression of genes important in UV-B photoprotection (eg. *EARLY LIGHT INDUCIBLE PROTEINS*, *PHOTOREACTIVATING ENZYME 1* and flavonoid biosynthesis genes) is apparently stimulated *via* a signalling pathway which requires both UVR8 and HY5. Accordingly, in the absence of either UVR8 or HY5 plants show an increased susceptibility to supplementary UV-B induced growth inhibition and leaf damage. Microarray analyses of UV-B induced gene expression patterns in both the *uvr8* and *hy5* mutants, compared to wild-type controls, have identified a subset of genes regulated by a UV-B signalling pathway in which UVR8 and HY5 each plays a critical role. The loss of this signalling pathway probably places the plant at a significant selective disadvantage in the wild.

In addition to this work, the *30e5* mutant which had previously been isolated, using a *CHS* promoter – *β-GLUCURONIDASE* (*GUS*) reporter gene, as deficient in its response to UV-B was characterized. Although *30e5* was subsequently found to accumulate *CHS* transcripts normally in response to UV-B and UV-A, it proved to be more sensitive to supplementary UV-B induced growth inhibition and leaf damage than was *uvr8*. Crosses with the corresponding mutants demonstrated that the *30e5* mutant gene is an allele of the *DIFFERENTIAL DEVELOPMENT OF VASCULAR-ASSOCIATED CELLS* (*DOV1*) gene. Map-based cloning delimited the *30E5* gene to a 40 kb region of *Arabidopsis* chromosome IV which contained 12-14 genes. One of these candidate genes, *ATase DEFICIENT 2* (*ATD2*), encodes 5-PHOSPHORIBOSYL-1-PYROPHOSPHATE AMIDOTRANSFERASE 2 (ATase2), an isoenzyme which catalyzes the first step of *de novo* purine biosynthesis in *Arabidopsis*. The *atd2* mutant is strikingly similar in phenotype to *30e5* and crosses are being initiated to determine whether *30e5* is an allele of the *ATD2* gene.

CHAPTER 1

RESPONSES TO LIGHT IN PLANTS

1.1 Introduction

Many photoreceptors make light work for *Arabidopsis*, but not for those employed to delineate the associated signal transduction networks. Light is the central factor in the normal development of plants and so the molecular events involved in the detection of, and response to, light by plants are critical for life on earth. It is somewhat surprising then, that our understanding of how plants respond to light lags significantly behind progress made in recent years in parallel fields such as pharmacology and medicine. Plants have sustained us in the past and hold the key to our future, so it would be unwise to neglect the opportunities which advances in biotechnology and genetics have presented for us to seek a more comprehensive grasp on how they perceive and react to light.

1.2 Light and Light-Driven Responses

1.2.1 The Nature of Light

The great Italian astronomer and mathematician Galileo Galilei died in the year 1642 and on Christmas Day of that same year, three months after the start of the English Civil War, Isaac Newton was born in Lincolnshire. By the time Newton entered Trinity College, Cambridge in 1661 it was well known that when light was passed through a prism it produced a spectrum of colours (Figure 1.1). However, it was commonly believed that the explanation for this phenomenon lay in the prism's ability to change the appearance of pure and indivisible white light. In 1669-70, Newton conducted a series of experiments during which he passed white light from a hole drilled in a window shutter through a prism and onto a card. Isolating the red and then blue light components produced by the prism using a hole in the card, Newton next passed the coloured light through a second prism and was able to show that no further change in the colour of light took place. In this, his *experimentum crucis* Isaac Newton demonstrated that white light was made up of pure constituents of individual colours refracted to differing degrees by the prism (Bradley et al., 2000, Muir, 1994).

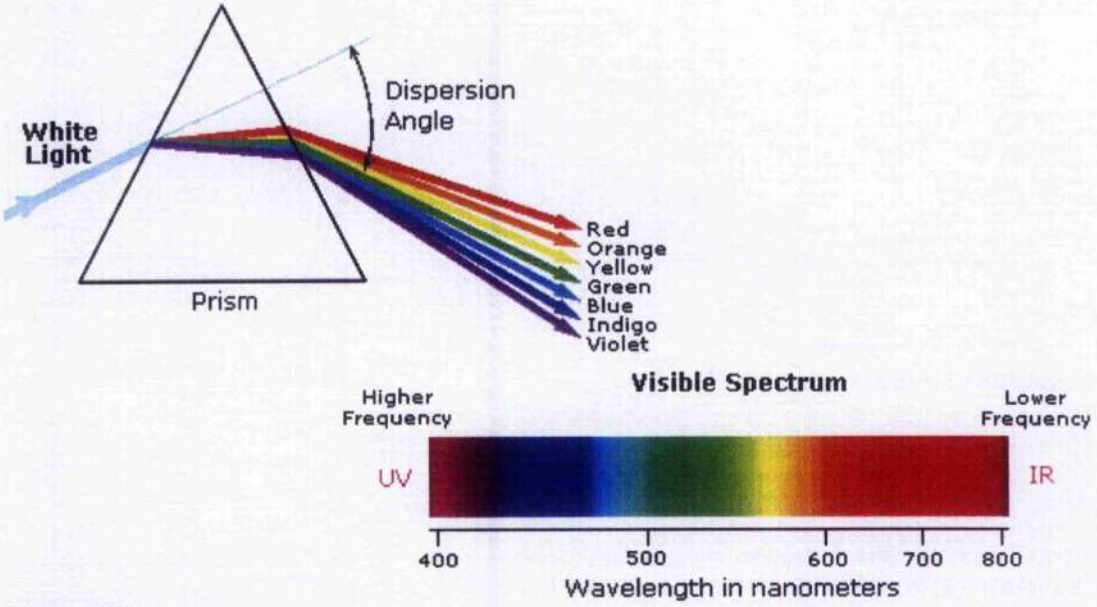


Figure 1.1
A prism separates white light into its component light qualities.
(Taken from Michigan State University Chemistry Department webpage, http://www.cem.msu.edu/~reusch/VirtualText/Spectrpy/UV-Vis/spectrum.htm).

Today physics appreciates that light is electromagnetic radiation which can be comprehensively described only by taking into account both its wave- and particle-like attributes (Aphalo, 2001). The different colours which Newton proved were distinct components of white light correspond to different, though somewhat arbitrarily defined, wavelengths: far red light has wavelength 700-800 nm, red light is 600-700 nm, blue is 390-500 and ultraviolet equates to wavelength 200-390 nm. The high-energy, and therefore potentially damaging, ultraviolet light can further be subdivided into UV-A (320-390 nm), UV-B (280-320 nm) and UV-C (200-280 nm).

Since they possess photoreceptors and signal transduction pathways which can be specifically activated by the different components of white light (Batschauer, 1998, Briggs and Christie, 2002, Lin, 2002, Quail, 2002b, Gyula et al., 2003), it is intriguing to think that higher plants have essentially been conducting Newton's *experimentum crucis* for millions of years in order to trigger a variety of responses. The known photoreceptors, crucial for perceiving light of different wavelength in higher plants, include the phytochromes, cryptochromes and phototropins. Whilst some of these photoreceptors are being rapidly characterized, others almost certainly await discovery and our knowledge of the associated signal transduction pathways which mediate the crucial responses in plants is far from complete.

1.2.2 Plants Have a Variety of Responses to Light

Life on Earth depends upon the ability of plants to detect and respond to light. In addition to the critical position plants occupy in the food chain, photosynthesis is the main source of atmospheric oxygen on which aerobic organisms rely (Bailey, 1999). The capacity of plants to capture light over a broad range of wavelengths for photosynthesis hinges on the co-action of several photoreceptor pigments (including chlorophylls and carotenoids) each of which has a characteristic absorption spectrum, retaining light of certain wavelengths better than that of others (Lack and Evans, 2001).

Plants also utilize light of wavelengths distinct from those primarily associated with photosynthesis, to trigger a variety of physiological and developmental responses (Ahmad, 1999, Fankhauser and Chory, 1997). Light activated responses, stimulated by far-red, blue and ultraviolet light, are critical throughout the life of a higher plant. From germination through to flowering, from cotyledon expansion to stomatal opening and entrainment of the circadian clock, certain physiological processes have been shown to depend particularly upon wavelengths of light at either end of the visible spectrum.

However, it has been possible to deduce, from a variety of simple studies, that there is an underlying complexity to the regulation of light activated responses in plants. Some responses, eg. the inhibition of hypocotyl elongation in *Arabidopsis*, are regulated almost equally well by a number of different light qualities (Khurana et al., 1998, Goto et al., 1993, Christie and Briggs, 2001). By contrast, in some responses one wavelength range or light quality plays a predominant role, for example UV-A / blue light in the regulation of phototropism (Baskin and Iino, 1987). Sometimes the intensity of the incident light is of critical importance to such an extent that the response in question can actually be reversed under high fluence rates, as is the case in the stimulation of chloroplast accumulation / avoidance by blue light in *Arabidopsis* (Kagawa and Wada, 2002, Jarillo et al., 2001). Studies on the light induced regulation of transcription of the key flavonoid biosynthetic enzyme CHALCONE SYNTHASE (CHS) have highlighted further idiosynchrasies. For example, the ability of far-red light to induce *CHS* gene expression in *Arabidopsis* is lost as the young seedlings develop, highlighting a developmental switch in the use of a particular light quality (Kaiser et al., 1995). Furthermore, in mature leaf tissue combinations of more than one light quality, for example when plants are irradiated with blue and UV-B light simultaneously, can hyperstimulate *CHS* expression (Fuglevand et al., 1996, Jenkins et al., 2001).

Nonetheless, although the integration of perceived light signals is a multifarious process, clearly plants are only able to effect distinctive responses to different light qualities because they are able to distinguish amongst incident red, far-red, blue and ultraviolet light.

Accordingly, in recent years a number of photoreceptors have been discovered which function as prime movers in the detection of particular light qualities.

1.3 Photoperception and Signal Transduction

1.3.1 Phytochromes

1.3.1.1 Introduction

Since its discovery in 1959-60 by the Beltsville group of USDA (Tong et al., 2000), the photoconvertible photoreceptor phytochrome's role in mediating responses to red and far-red light in higher plants has drawn much attention. The responses in which phytochrome, which is almost certainly ubiquitous in higher plants (Wang and Deng, 2003), plays an important role include germination, de-etiolation, shade avoidance, flowering, chloroplast development, cell growth, gene expression and regulation of the circadian clock (Kendrick and Kronenberg, 1994, Soh et al., 2000, Fankhauser, 2001, Kevei and Nagy, 2003). The 120-130 kDa phytochrome monomers bind a linear tetrapyrrol chromophore (phytochromobilin) and form a P_R homodimer which, on absorption of red light, is converted into the P_{FR} (far-red sensitive) form that is considered physiologically active for most responses (Casal, 2000 , Song, 1999). Phytochrome is probably best known to biology students the world over as the photoreversible photoreceptor triggering or delaying physiological and developmental processes depending on whether the final flash of light to which a plant is exposed is of the red or far-red variety (Campbell et al., 1999). However, the capacity to stimulate phytochrome is not limited solely to long wavelength light. For example, in addition to the absorption maximae, for P_R in the red (660 nm) and P_{FR} in the far-red (730 nm) wavelength range, phytochrome can also mediate responses to blue and ultraviolet light (Fankhauser, 2001, Buchanan et al., 2000, Lin, 2000).

1.3.1..2 Phytochromes as Light-Regulated Protein Kinases

The phytochromes have two carboxyl-terminal structural domains called the PAS repeat and the histidine kinase-related domain. The PAS repeat domain was believed to interact with other signalling components (Krall and Reed, 2000, Bibikov et al., 2000), whilst the discovery of cyanobacterial phytochrome histidine kinases fuelled forty-year old speculation that eukaryotic phytochromes are actually enzymes. Further work provided a more solid foundation for the hypothesis that higher plant phytochromes are, in fact, light-regulated serine / threonine protein kinases (Maheshwari et al., 1999, Yeh and Lagarias, 1998, Fankhauser and Chory, 1999, Fankhauser, 2000). In addition, it was discovered that the light-dependent nuclear import of phytochromes may play a key role in regulating responses (Nagy et al., 2000, Reed, 1999, Nagy and Schafer, 2000, Hudson, 2000). Curiously however, the C-terminal region (containing signalling motifs such as the kinase domain) of *Arabidopsis* phyB has recently been shown to hinder, rather than stimulate, the activity of the photoreceptor (Matsushita et al., 2003). Perhaps then, it is the N-terminal domain of phyB which transduces the phytochrome signal downstream. Clearly, further research will be required to gain a better understanding of the molecular basis of phytochrome action.

1.3.1.3 *Arabidopsis* Phytochromes

There are five phytochromes in *Arabidopsis thaliana* (phyA – phyE) of which phyA (light labile and mediating mainly far-red responses) and phyB (light stable and mediating mainly responses to red light) appear to be the most important (Sineshchekov et al., 1999, Hennig et al., 2000, Reed et al., 2000). *Arabidopsis* phyC and E have different spectral characteristics from phyA and B (Eichenberg et al., 2000), whereas phyB and phyD, at approximately 80% identical, are products of a recently duplicated gene. phyD is important in the shade-avoidance syndrome (Devlin et al., 1999). Only light-labile phyA is classified as a type I phytochrome in *Arabidopsis* and, interestingly, this plays a significant role in gene expression and germination in response to low intensity light over a much broader range of wavelengths than is generally seen in other photoreceptor mediated responses. PhyA also mediates

responses such as de-etiolation and *CHS* expression in *Arabidopsis* seedlings which are stimulated by far-red light, unlike most other phytochrome responses which are red light induced. Phytochromes B-E are categorized as type II due to their light-stable nature and these are generally responsible for the classical red / far-red reversible responses (Kaiser et al., 1995, Fankhauser, 2001).

1.3.1.4 Phytochrome Signal Transduction

Much work is currently underway to try to uncover the molecular events which take place between the light induced stimulation of phytochrome and the plant responses which result. Phytochrome mediated control of gene expression appears to involve the photoreceptor molecules in binding to transcription factors once inside the nucleus. In addition to a multitude of putative signalling components (Wang and Deng, 2003, Kevei and Nagy, 2003, Schafer and Bowler, 2002, Quail, 2002b), general second messengers such as heterotrimeric G-proteins, cGMP and calcium have been implicated in phytochrome signalling pathways by pharmacological studies in tomato (Mustilli and Bowler, 1997, Fankhauser, 2001). One signal transduction component involved in the phytochrome signalling pathway and attracting a great deal of interest is the phytochrome interacting factor 3 (PIF3). PIF3 is a basic-helix-loop-helix (bHLH) protein which appears to bind phyB (in the activated P_{FR} form) after the photoreceptor has undergone light induced nuclear import, playing a key but complex role in phytochrome responses. It is possible that a related bHLH transcription factor, RSF1 (also known as HFR1 or REP1), plays an analogous role in phyA signalling (Zhu et al., 2000, Ni et al., 1998, Kim et al., 2003, Fankhauser, 2001). The positive regulator of photomorphogenesis HY5 has also been implicated downstream of phytochrome stimulation (Chattopadhyay et al., 1998, Saijo et al., 2003, Fankhauser, 2001).

1.3.2 UV-A / Blue Light

1.3.2.1 The Cryptochromes

1.3.2.1.1 cry1, cry2 and cry3

Photoreceptors active in responses to UV-A / blue light include the *Arabidopsis* cryptochromes, cry1 (HY4) and cry2 (FAH1 / PHH1), which are similar in structure to the prokaryotic DNA photolyases from which they probably evolved. However, although cryptochromes are flavoproteins with significant N-terminal sequence identity to their presumed ancestors, they lack photolyase activity - implying that they fulfil a novel role in plants (Guo et al., 1998, Hoffman et al., 1996, Briggs and Huala, 1999, Kleiner et al., 1999, Cashmore et al., 1999, Ahmad et al., 1998a). Both cry1 and cry2 are involved in blue light-dependent responses including inhibition of hypocotyl elongation, leaf and cotyledon expansion, pigment biosynthesis, stem growth, internode elongation and control of flowering time (Ninu et al., 1999). Although there is a significant overlap in the functions of cry1 and cry2 in *Arabidopsis* (eg. cry2 can partially compensate for the loss of cry1 in some UV-A / blue responses), there are important differences too. One distinction between the cryptochrome photoreceptors is that whilst cry1 is the principal photoreceptor involved in UV-A / blue light mediated inhibition of hypocotyl elongation in seedlings and *CHS* expression in mature leaf tissue, cry2 has the more important role to play in regulating when a plant will flower. Unlike cry1, cry2 is also subject to degradation under high intensity blue light, underlining the fact that the latter's utility often seems to be limited to low fluence blue light responses. Both cryptochromes are, however, involved in providing blue light input to the circadian clock (Yanovsky et al., 2001). Although much research has been directed towards unravelling the mechanism(s) by which light activated cryptochromes mediate responses in higher plants, clear and unequivocal answers remain elusive (Bouly et al., 2003, Jenkins et al., 2001, Lin, 2000, Christie and Briggs, 2001). A third *Arabidopsis* putative photoreceptor (Atcry3), closely related to a cryptochrome found in the cyanobacterium *Synechocystis*, has recently been discovered. Like the other cryptochromes Atcry3 has no photolyase activity but binds flavin adenine dinucleotide (FAD). Additionally Atcry3 appears

to be able to bind to DNA, and it has an N-terminal sequence which might facilitate its localization in chloroplasts and mitochondria (Kleine et al., 2003).

1.3.2.1.2 Cryptochrome Signal Transduction

Because these are similar in sequence to photolyases which repair pyrimidine dimers by transferring electrons to the damaged DNA, it has long been thought possible that the cryptochromes may somehow also mediate responses to UV-A / blue light by electron transfer. However, cry1 and cry2 possess distinctive C-terminal extensions not found in the photolyases which appear to be necessary (Ahmad et al., 1995) and sufficient (Yang et al., 2000) for initiating signalling, probably by the same mechanism (Ahmad et al., 1998a). So perhaps the N-terminal domain acts to repress cryptochrome signal transduction until illumination with UV-A / blue light releases the C-terminal domain (Christie and Briggs, 2001).

In addition, the blue light induced inhibition of hypocotyl elongation in *Arabidopsis* and other plant species, is believed to involve the cry1 mediated activation of a cellular anion channel, producing a transient depolarization of the plasma membrane, as part of the slow (as distinct from the rapid) phase of the hypocotyl growth inhibition response (Spalding, 2000, Cho and Spalding, 1996, Parks et al., 1998, Christie and Briggs, 2001).

However, as highlighted above, the mechanism(s) by which cryptochromes generate responses to illumination is uncertain despite the many studies which have been done. Most recently, evidence has been obtained which indicates that phosphorylation of cryptochromes may be an important step in controlling their regulation of responses to blue light (Lin and Shalitin, 2003, Shalitin et al., 2003); hopefully a clearer picture of how these photoreceptors mediate signal transduction will emerge over the next few years.

1.3.2.2 The Phototropins

1.3.2.2.1 phot1 and phot2

The phototropic response is one of the most important rejoinders a young plant makes to incident light. Phototropism is a process of reorientation in response to lateral differences in light quality or quantity and its importance lies in the fact that it allows plants to optimize their photosynthetic efforts by adopting the most illuminated disposition. Blue and UV-A are the principal light qualities involved in the higher plant phototropic response, and mutant studies combined with sequence comparisons have identified two of the critical photoreceptors which mediate phototropism in *Arabidopsis* together with homologues from other species. The photoreceptors, collectively known as the phototropins, have been designated phot1 (originally called nph1 – non phototropic hypocotyl) and phot2 (originally called npl1 – nph1 like) and they bear a marked resemblance to one another from both a structural and photochemical perspective (Christie and Briggs, 2001, Kanegae et al., 2000, Christie et al., 1999, Kagawa et al., 2001, Crosson and Moffat, 2001).

1.3.2.2.2 Phototropin Photochemistry and Signal Transduction

A combination of studies have indicated that the 120 kDa, 996 amino acid *PHOT1* gene product is a dual-chromophoric flavoprotein and autophosphorylating ser-thr protein kinase activated by UV-A / blue light which associates with, but doesn't traverse, the plasma membrane (Christie and Briggs, 2001, Huala et al., 1997, Liscum and Briggs, 1995, Reymond et al., 1992, Short and Briggs, 1994, Briggs and Huala, 1999). The phot1 polypeptide N-terminus contains 2 PAS domains designated LOV (because these are found in a group of proteins involved in sensing light, oxygen or voltage). The PAS domains, LOV1 and LOV2, each encapsulate a tightly held flavin mononucleotide (FMN) chromophore and they mediate light perception by undergoing a photocycle where the initial event is the formation of a temporary covalent bond between a conserved cysteine residue within the LOV domain and the FMN chromophore. Somehow, the formation of the flavin-cysteinyl adduct produces a conformational change in the flavin which, in turn, changes the shape of the phot1 protein

itself. In phot1 the LOV2 domain is essential for phototropism and the activity of the kinase but LOV1, by contrast, seems not to be directly involved in phototropism. Perhaps the LOV1 domain plays a structural or regulatory role within the phot1 photoreceptor kinase but at present its function is something of a mystery (Briggs and Olney, 2001, Briggs and Huala, 1999, Christie et al., 1999, Salomon et al., 2000, Maheshwari et al., 1999, Christie and Briggs, 2001). The signalling mechanism by which phot1 ultimately alters the degree of curvature in a seedling is not known either, but there is evidence that both the second messenger calcium and phytohormone auxin play important roles in generating the visible phototropic response (Lin, 2000, Baum et al., 1999, Liscum and Stowe-Evans, 2000, Christie and Briggs, 2001). The domain organization of some plant photoreceptors is shown below (Figure 1.2).

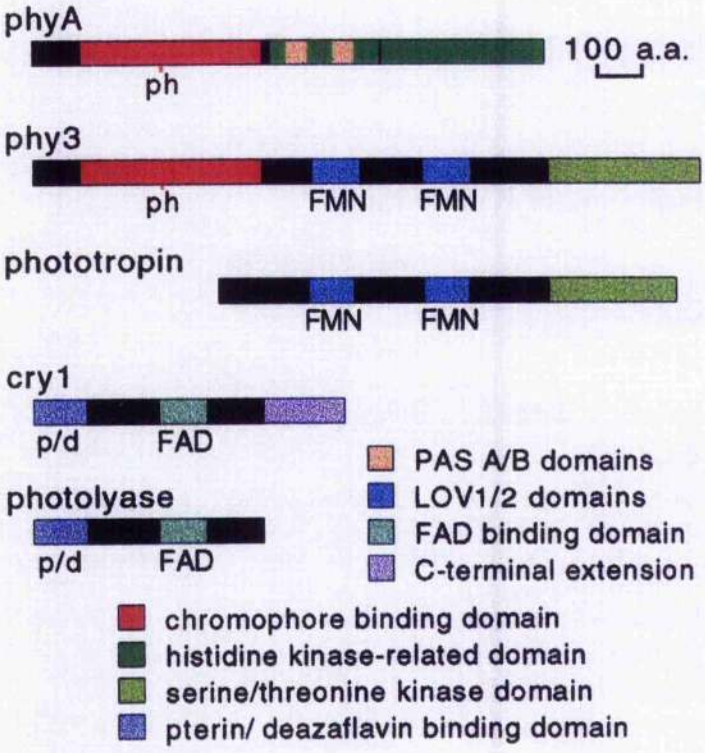


Figure 1.2
Domain organization of the three classes of plant photoreceptors and a typical prokaryotic photolyase. PhyA, *Arabidopsis* phytochrome A; Phy3, *Adiantum capillus-veneris* phy3; Phototropin, *Arabidopsis* phototropin (nph1); cry1, *Arabidopsis* cry1 (Taken from Briggs and Olney, 2001).

1.3.2.2.3 Other Responses Mediated by the Phototropins

Since *Arabidopsis* phot1 null mutants, such as *phot1-5* (Kinoshita et al., 2001), lack phototropic curvature in response to low (1 μmol $m^{-2}s^{-1}$) but not high fluence (100 μmol $m^{-2}s^{-1}$) blue light at least one more photoreceptor must contribute to phototropism in *Arabidopsis* (Sakai et al., 2000, Christie and Briggs, 2001), and the phot2 protein appears to fulfil this complementary role. *Arabidopsis* phot2 is almost identical to phot1 in structure and photochemistry. Both photoreceptors are involved in phototropism, chloroplast relocation and stomatal opening. However, in phot2 the photosensitivity of the LOV2 domain is only twice as great as that of LOV1, whereas in phot1 LOV2 is ten times as photosensitive as the LOV1 domain. The involvement of phot1 and phot2 in blue light stimulated chloroplast movement is curious in so far as both photoreceptors can stimulate chloroplast accumulation at the illuminated surface of a plant cell, but only phot2 can mediate the protective avoidance (or shade-seeking) response to prevent damage to the photosynthetic machinery when the light intensity becomes too high (Jarillo et al., 2001, Kagawa et al., 2001, Christie and Briggs, 2001). Perhaps of related significance is the fact that phot2 only mediates phototropism at high fluence rates. In fact, phot1 responds to lower fluence rate blue light than does phot2 in both phototropism and chloroplast relocation. In view of the fluence rate specificity that the phototropins display in mediating phototropism and chloroplast movement, it may come as a surprise to learn that although the *phot1* and *phot2* mutants show little reduction in blue light induced stomatal opening, *phot1phot2* shows almost a complete loss of the response. So phot1 and phot2 appear to act in a strictly redundant fashion as blue light receptors mediating stomatal opening in contrast to the complementary roles they play in regulating other responses (Kinoshita et al., 2001).

1.3.2.2.4 Other Photoreceptors and Pigments Implicated in Phototropin Responses

Other photoreceptors, such as cryptochromes and phytochromes have been implicated in modulating phototropism, although it now seems less likely that these play a primary role in mediating the response (Ahmad et al., 1998b, Lasceve et al., 1999, Janoudi et al., 1997a, Janoudi et al., 1997b, Christie and Briggs, 2001). It should be noted in addition, that inhibitor and mutant studies have been done which indicate that the xanthophyll carotenoid, zeaxanthin may be involved in mediating stomatal opening in response to blue light (Talbott et al., 2003, Bailey, 1999, Zeiger and Zhu, 1998, Frechilla et al., 1999, Frechilla et al., 2000). Future studies should provide a clearer understanding of what, if any, role zeaxanthin plays in the phototropin responses.

1.3.3 UV-B

1.3.3.1 Introduction

Ultraviolet-B is UV light of short wavelength (280-320 nm) and so it carries a great deal of energy. Fortunately for us, most solar UV-B, along with all solar UV-C, is absorbed by atmospheric gases such as ozone. By contrast, UV-A from the sun is allowed to reach the Earth's surface largely unimpeded (Frohnmeyer and Staiger, 2003). Over the last few decades however, the concentration of stratospheric ozone has been significantly diminished as a result of mankind's misuse of his environment.

The consequence of less ozone above our planet has been a concomitant increase in the quantity of UV-B which reaches the Earth's surface - although there is disagreement over how serious the implications of increased levels of UV-B radiation will turn out to be (Allen et al., 1998, Paul, 2000, Wilhelmova, 2001, Zaller et al., 2002, Frohnmeyer and Staiger, 2003, Newsham, 2003, Brosche and Strid, 2003, Stratmann, 2003). Good grounds to doubt that there will be an ecological catastrophe as a result of stratospheric ozone depletion are given by the fact that environmental UV-B fluence rates vary regularly and to a much greater extent for entirely different reasons, such as variations in latitude, time of day, time of year, elevation, angle of the sun and cloud cover (Lubin and Jensen, 1995, Madronich et al., 1998, Paul, 2000, L' Hirondelle and Binder, 2002). Additionally, plants have demonstrated that they have the plasticity to adapt and tolerate high doses of UV-B (Jordan, 1996, Frohnmeyer and Staiger, 2003, Torabinejad and Caldwell, 2000).

Nonetheless, there is no doubt that increased levels of ambient UV-B can have a deleterious effect on plant growth and yield for many species and such effects, even if small on a local scale, could be significant globally (Stratmann, 2003). For an important crop such as rice, which is a staple food for half the world's population (Adam, 2000), we cannot afford to take the implications of a possible global reduction in yield lightly (Ziska, 1996, Hidema et al., 1997, Kumagai et al., 2001, He et al., 1993, Allen et al., 1998) and it is therefore essential that strategies are developed for dealing with potential difficulties before they are realized. In the light of these changing circumstances, understanding how higher plants perceive and respond

to UV-B should be considered a priority if we want to be in a position to solve problems created by an increase in tropospheric UV-B.

1.3.3.2 UV-B Responses in Higher Plants

An increase in the ambient UV-B fluence rate will retard the growth of many plant species, reducing overall biomass and crop yield whilst generating thicker leaves; fertility may also be reduced. Indeed, UV-B can exert a variety of physical, developmental and morphological effects on plants and many responses appear to involve the plant becoming damaged, or trying to avoid becoming damaged by the incident, high energy UV light. For example direct damage to DNA, involving the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4 PPs), occurs at the same time as UV-B stimulates DNA repair mechanisms such as homologous recombination. Damage can also occur to lipid membranes, proteins and components of the photosynthetic apparatus such as chlorophyll, carotenoids and the D1 and D2 photosystem II proteins.

UV-B also stimulates the expression of genes involved in phenylpropanoid biosynthesis thus mediating the accumulation of UV-protective pigments (eg. flavonoids and hydoxycinnamate esters) in epidermal cell vacuoles, in an effort to prevent penetration of damaging UV-B into the underlying photosynthetic tissue (see Figure 1.3). Other stress responses triggered include the formation of reactive oxygen species (ROS) and the upregulation of antioxidant enzymes such as superoxide dismutase and ascorbate peroxidase together with the accumulation of PR-1 (Jansen et al., 1998, Caldwell et al., 2003, Frohnmeyer and Staiger, 2003).

However, there appears to be another, very different, aspect to the action of UV-B on higher plants and one which manifests itself in the responses of vulnerable seedlings to this high energy radiation. Although usually controlled by phytochromes and cryptochromes, UV-B appears to be capable of promoting photomorphogenesis (the onset of a plant's developmental program when first emerging into the light) in etiolated seedlings, inhibiting hypocotyl elongation and triggering the expansion of cotyledons (Kim et al., 1998). Curiously, there is

also evidence that UV-B wavelengths have the capacity to induce a phototropic response from etiolated seedlings, a reaction more commonly associated with UV-A / blue light and the phototropin photoreceptors (Shinkle et al., 1999).

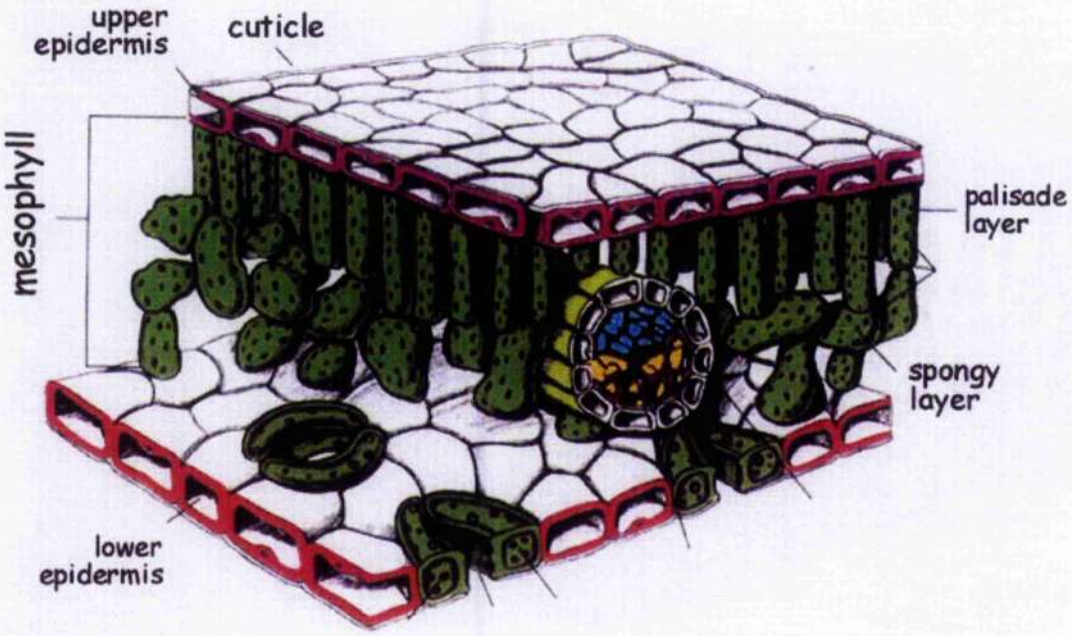


Figure 1.3
Cross section of an angiosperm leaf showing the epidermal cell layers and the underlying photosynthetic tissue (Taken from Lubey, Steve. Lubey's Biohelp – Photosynthesis. http://www.borg.com/~lubehawk/photosyn.htm).

1.3.3.3 UV-B has both Positive and Negative Effects on Plants

The first challenge then, when studying UV-B responses in higher plants, is to try to make sense of the different types of response which this particular light quality evokes. On the one hand, high energy UV-B has the capacity to do structural damage to the biomolecular architecture of plants, and plants try to respond in such a way as to minimize this damage. On the other hand, UV-B can elicit positive developmental responses, notably those which one might expect of an etiolated seedling switching from its dark growing developmental mode (skotomorphogenesis) to a light growing developmental mode (photomorphogenesis). It seems almost as if UV-B is mediating opposing (positive and negative) responses which suggests that two or more UV-B signal transduction pathways are operating in higher plants.

The situation becomes more complex when one considers the fact that these 'opposing' responses might be stimulated simultaneously by UV-B. When Kim *et al.* plotted the inhibition of hypocotyl elongation in wild-type *Arabidopsis* seedlings irradiated with UV-B against fluence rate the result was a biphasic curve (Figure 1.4). The implication of Kim *et al.*'s study, which also examined root growth and cotyledon expansion under varying UV-B fluence rates, appeared to be that at low UV-B light intensities (< 1 $\mu Em^{-2}s^{-1}$) photomorphogenesis was being stimulated whilst at high fluence rates (> 1 $\mu Em^{-2}s^{-1}$) the wild-type seedlings were responding in a manner consistent with their being damaged (Kim et al., 1998). So the intensity of incident UV-B light may be important in determining whether predominantly positive or negative responses are being stimulated in plants.

In addition to the positive photomorphogenetic and the negative manifestations of biomolecular damage there appears to be a third class of plant responses to UV-B, namely those responses which are designed to prevent or repair UV-B induced biomolecular damage. Such responses include the synthesis of phenylpropanoid derived pigments which can block the penetration of UV-B, the upregulation of antioxidant enzymes to deal with damaging ROS produced by UV-B induced photo-oxidative stress, downregulation in production of proteins (such as some involved in photosynthesis) particularly vulnerable to UV-B and the

upregulation of *Arabidopsis* DNA repair genes such as *PHR1* and *RAD51* (Brosche and Strid, 2003, Ries et al., 2000).

Revealingly, the anonymous reviewer of a recent paper published on a genetic approach to understanding UV-B signalling in *Arabidopsis* stated that "A strong response to UV-B indicates a UV-resistant or UV-sensitive plant, depending on your point of view." Thus, because of the differing nature of the various plant responses to UV-B described above and in order to preserve clarity in the field, it is probably best to describe the magnitude of each response very specifically rather than commenting vaguely on UV-B sensitive or insensitive plants.

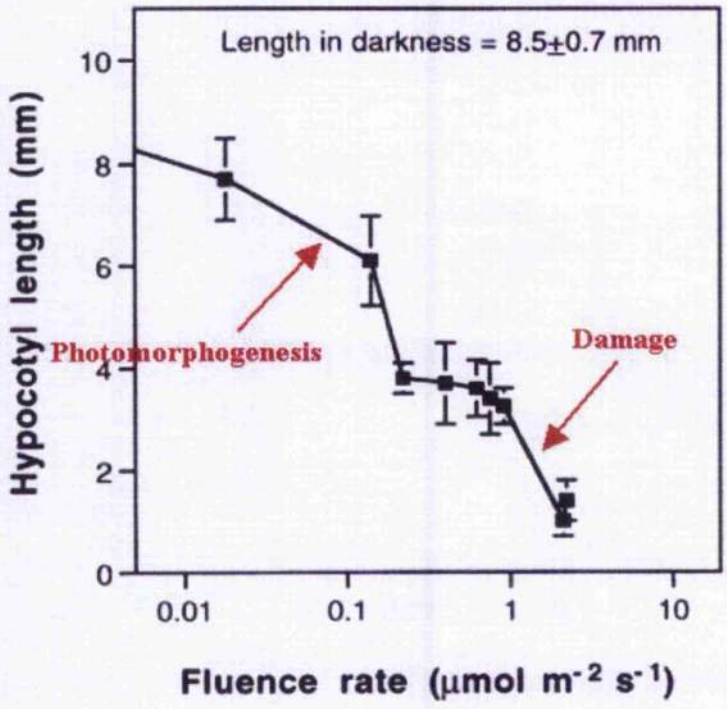


Figure 1.4
Inhibition of hypocotyl elongation in 5-day-old wild-type L. *erecta* seedlings suggest there may be more than one category of response (depending on fluence rate) to UV-B irradiation (Modified from Kim et al., 1998).

1.3.3.4 UV-B Photoperception

1.3.3.4.1 Introduction

It seems clear then, from the variety of responses and even the type of response elicited, that higher plants have more than one way of perceiving UV-B light. Because there is significant overlap between the signalling intermediates involved in high fluence UV-B and stress response pathways (Izaguirre et al., 2003, Jenkins et al., 2001, Frohnmeyer and Staiger, 2003), it seems reasonable to suggest that UV-B may here be acting not so much as a source of illumination but as an agent of physical violence on the plant. Perhaps a plant's response to high fluence UV-B is essentially a general reaction to stress or mechanical damage.

Since not all plant responses to UV-B are derived from biomolecular damage, however, it is also possible that plants possess a photoreceptor which is principally stimulated by UV-B (Kim et al., 1998, Barnes et al., 1996, Shinkle et al., 1999). It would be sensible, from an adaptive perspective, for plants to be able to prepare for assault with damaging UV-B by utilizing photoreception as an early warning system rather than simply trying to deal with tissue damage after it occurs. Since it is known that phytochromes, cryptochromes and phototropins all mediate photomorphogenetic responses which can also be stimulated by UV-B in the absence of these photoreceptors (Suesslin and Frohnmeyer, 2003, Kim et al., 1998, Shinkle et al., 1999) it seems likely that higher plants possess a UV-B photoreceptor which may likewise exhibit typical chromophore dependent photochemistry. Such a photoreceptor(s) may be capable of detecting low fluence UV-B ($< 1\ \mu Em^{-2}s^{-1}$) but might also mediate responses to UV-B which extend beyond photomorphogenesis.

Revealingly, many of the UV-B signalling components which are shared by other stress response pathways (specifically the wounding and pathogen-defence pathways) have been shown to play no role in the UV-B induction of *CHS* gene expression (Jenkins et al., 2001). Furthermore, action spectra for flavonoid and anthocyanin biosynthesis from various plants show activity between 290 and 300 nm which would be consistent with the function of a UV-B photoreceptor containing a flavin or pterin chromophore (Ensminger and Schafer, 1992, Galland and Senger, 1988, Brosche and Strid, 2003).

1.3.3.4.2 Phytochromes, Cryptochromes and Phototropins in UV-B Signalling

The possibility that known photoreceptors are responsible for mediating some plant responses to UV-B has been acknowledged for many years. In the past, comparisons of action and absorption spectra have been used to identify cellular components involved in a particular response (Briggs and Liscum, 1997, Horwitz and Berrocal, 1997, Ahmad et al., 2002). However, establishing whether or not existing photoreceptors mediate UV-B responses is complicated by the fact that the peak(s) in an action spectrum for any particular plant response need not be derived from a single photoreceptor. Additionally, all proteins (due to their aromatic amino acid content) absorb UV-B with a maxima at around 280 nm – although it should be noted that very few, if any, proteins can initiate photochemistry solely as a result of the ability of their amino acids to absorb light (J. M. Christie, personal communication).

Some phytochromes (eg. *Arabidopsis* phyA) are known to have the capacity to absorb light over a very broad range of wavelengths and to mediate particular responses, such as germination, as a result (Batschauer et al., 1996, Buchanan et al., 2000). In addition, the action spectrum for alfalfa phototropism displays a peak at around 280 nm (Figure 1.5), although it is not clear whether this peak results from the action of either phototropin (Baskin and Iino, 1987). By contrast, it is not thought that the cryptochromes play a significant role in detecting UV-B (Jenkins et al., 2001).

In general, current evidence strongly suggests that whilst phytochromes and cryptochromes play an important role in adjusting some plant responses to UV-B they are not the primary photoreceptors by which the UV-B signal is perceived. For example, although phyA and phyB certainly play an important role in the inhibition of hypocotyl elongation in *Arabidopsis* seedlings, and it remains possible that these act redundantly as primary UV-B photoreceptors (Kim et al., 1998), more recent evidence suggests that UV-B can stimulate this response without these phytochromes (Suesslin and Frohnmeyer, 2003, Frohnmeyer and Staiger, 2003). Similarly, whilst phyB has been shown to negatively regulate UV-B induced *CHS* expression in mature *Arabidopsis* leaf tissue, single, double and triple photoreceptor mutant studies strongly suggest that neither phytochromes nor cryptochromes (cry1 or cry2) are the

primary photoreceptors for this response (Wade et al., 2001). The fact that, in contrast to the situation in seedlings, mature *Arabidopsis* leaf tissue shows no *CHS* expression in response to red or far red light also indicates that the UV-B pathway here is not dependent on phytochrome; additionally, the distinctive kinetics of the UV-A / blue pathway suggests further that the cryptochromes are not required for UV-B induced *CHS* expression (Wade et al., 2001, Brosche and Strid, 2003, Christie and Jenkins, 1996, Jenkins et al., 2001).

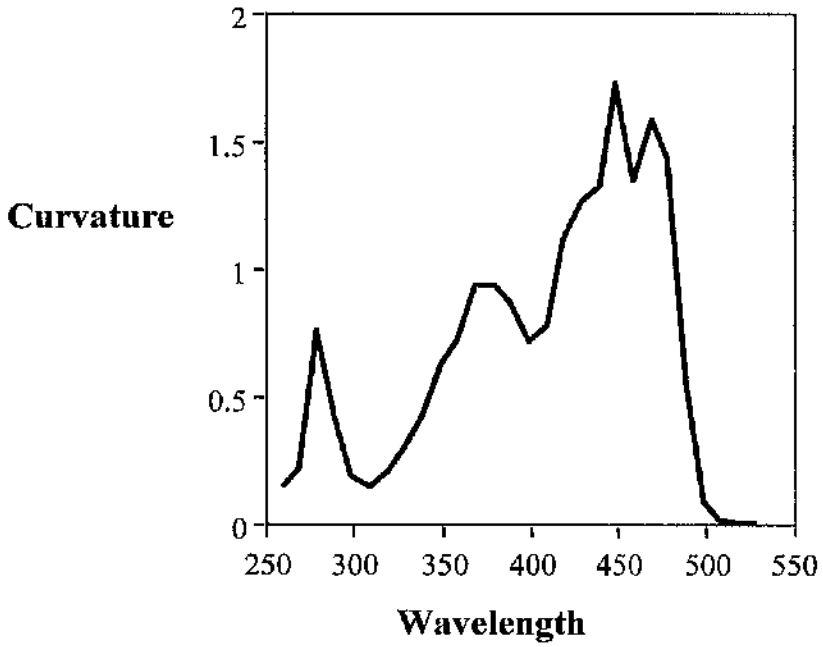


Figure 1.5
The action spectrum for alfalfa phototropism shows that UV-B can induce a phototropic response (Taken from Baskin and Iino, 1987).

1.3.3.4.3 The Role of Damage to DNA in UV-B Signalling

Since a clear precedent for such a mechanism has been set in animal cells, one intriguing possibility is that damage to nuclear DNA might be the initial event in a UV-B signal transduction pathway in plants (Bender et al., 1997, Jenkins et al., 2001). A very simple example of UV-B induced DNA damage stimulating a cellular response in plants is perhaps the repair of damaged DNA itself by a process called nucleotide excision repair (NER) which detects distortions in the DNA helix produced by, for example, pyrimidine dimer formation. Although plant photolyases (enzymes homologous to the cryptochrome photoreceptors which probably repair most pyrimidine dimers) may be activated directly by light, there is good evidence that NER also occurs in plants (Buchanan et al., 2000, Liu et al., 2000, Gallego et al., 2000). It is also known that DNA damage can be produced by UV-B treatments which induce *CHS* expression in plants (Jenkins et al., 1995).

However, work by Conconi *et al.* on tomato gene expression (Conconi et al., 1996), together with the fact that blue light (which promotes DNA repair) hyperstimulates UV-B induced *CHS* expression in *Arabidopsis* (Fuglevand et al., 1996), has shown that for many (but not all (Kucera et al., 2003)) responses, plants do not mediate UV-B induced gene expression *via* a nuclear DNA damage signal (Brosche and Strid, 2003, Jenkins et al., 2001). Furthermore, whilst it is known that DNA absorbs light of wavelength 260 nm most strongly and pyrimidine dimers are produced in the greatest quantities by 280 nm light (Chavaudra, 1979), action spectra of plant UV-B responses generally peak between 290 and 310 nm with shorter wavelength light often exerting a negative influence (Herrlich et al., 1997). In addition, there appears to be a lack of correlation (eg. of causative wavelength) between UV-B induced DNA damage and the relevant transcript profile changes (Kim et al., 1998, Frohnmeyer et al., 1999, Kalbin et al., 2001, Frohnmeyer and Staiger, 2003).

There also appears to be no relationship between UV-B induced photomorphogenesis in *Arabidopsis* seedlings and the production of DNA damage. Not only does low fluence rate UV-B light ($< 1\ \mu Em^{-2}s^{-1}$) appear to be capable of stimulating both photomorphogenesis and flavonoid biosynthesis without generating detectable CPD formation (Frohnmeyer and

Staiger, 2003), but *Arabidopsis* mutants which have been shown to be hypersensitive to UV-B induced biomolecular damage (*tt5*, *uvr2* and *vtc1*) showed no increase, when compared with wild-type seedlings, in the extent of hypocotyl elongation inhibition at such low UV-B fluence rates (Kim et al., 1998).

1.3.3.4.4 Is There a UV-B Photoreceptor?

Whilst biomolecules, such as DNA and proteins, with aromatic moieties absorb UV-B (benzene rings with one or two nitrogen atoms absorb particularly well (Chavaudra, 1979)) a putative UV-B photoreceptor might reasonably be expected to possess a separate chromophore and it has been suggested that a flavin or pterin group would be consistent with the chromophore role (Ensminger and Schafer, 1992, Galland and Senger, 1988, Brosche and Strid, 2003). In addition, a number of separate observations indicate that UV-B can trigger cell signalling pathways which do not depend primarily on known photoreceptors or biomolecular damage (Brosche and Strid, 2003). Boccalandro *et al.* have also found that low doses of UV-B, perceived by something other than phytochromes, cryptochromes or DNA, enhance the phyB dependent expansion of cotyledons in etiolated *Arabidopsis* seedlings (Boccalandro et al., 2001). Thus, it seems likely that one or more unknown photoreceptors capable of transducing a low fluence UV-B signal to produce responses such as photomorphogenesis and flavonoid biosynthesis await discovery.

1.3.3.5 UV-B Signal Transduction

1.3.3.5.1 Introduction

Higher plants apparently have different mechanisms for perceiving UV-B light and transducing the perceived signal to produce appropriate responses. Some of these cell signalling pathways can be activated by stimuli other than UV-B and may be mediated by known photoreceptors or general detectors of plant stress. Nonetheless, it seems reasonable to suggest that plants possess photoperception systems and signal transduction pathways which are primarily sensitive to UV-B light (Brosche and Strid, 2003). Such UV-B specific

pathways may well be capable of stimulating photomorphogenesis, phototropism and flavonoid biosynthesis in *Arabidopsis*, particularly at low UV-B fluence rates.

Although little is known about the pathways which mediate UV-B signalling in higher plants, a number of second messengers and transcription factors / protein regulators have been implicated in such pathways. One possible, if much simplified, model for UV-B mediated signal transduction is illustrated below (Figure 1.6).

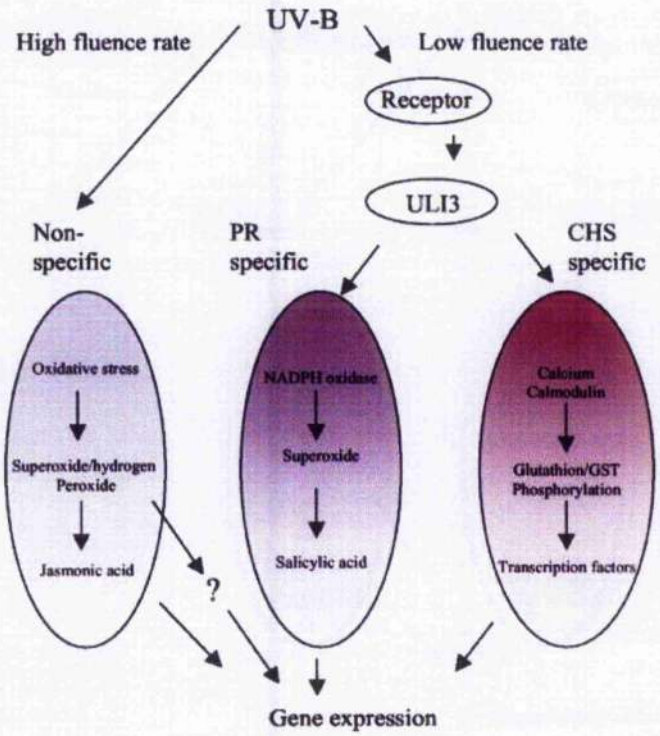


Figure 1.6
Proposed model for UV-B-mediated signal transduction.
PR, Pathogenesis-related protein; GST, glutathione *S*-transferase; ULI3, protein isolated from a UV-light-insensitive *Arabidopsis* mutant (Taken from Frohnmeyer and Staiger, 2003 which was, in turn, modified from Brosche and Strid, 2003).

1.3.3.5.2 Calcium and Calmodulin

Studies from a number of sources have converged on the conclusion that calcium is an important second messenger involved in upregulating *CHS* expression in higher plants such as *Arabidopsis*, parsley and soybean, in response to UV-A and UV-B - whilst the calcium binding protein calmodulin appears to play an important role in the UV-B *CHS* induction pathway only (Christie and Jenkins, 1996, Frohnmeyer et al., 1998, Frohnmeyer et al., 1999, Jenkins et al., 2001, Brosche and Strid, 2003). Further evidence has suggested that removal of calcium from the cytosol by specific Ca^{2+}ATPases may be an important step in upregulating *CHS* expression, and that the UV-B pathway may depend upon a calmodulin sensitive Ca^{2+}ATPase (Long and Jenkins, 1998, Jenkins et al., 2001).

1.3.3.5.3 Reactive Oxygen Species

Reactive oxygen species (ROS) can be regarded as signalling molecules produced by plants in response to UV-B, and may either be non-specific products of light induced stress or specific signals produced by enzymes - or indeed both (Brosche and Strid, 2003). UV-B certainly produces an increase in ROS accumulating in *Arabidopsis* and tobacco, and this results in a variety of changes in gene expression (Allan and Fluhr, 1997, Surplus et al., 1998, Mackerness et al., 2001, Brosche and Strid, 2003). For example, Mackerness *et al.* found that in *Arabidopsis* UV-B can activate NADPH oxidase and / or peroxidases producing ROS which alter gene expression, including the expression of some genes involved in photosynthesis (Mackerness et al., 2001, Brosche and Strid, 2003). It also seems likely that ROS generated simply as a non-specific reaction to plant tissue damage by high fluence UV-B are involved in regulating the expression of other genes, such as *PR-1* (Brosche and Strid, 2003). However, evidence from a variety of studies has strongly indicated that the UV-B induction of *CHS* expression in *Arabidopsis* does not depend upon ROS regardless of source and so it seems that *at least* two distinct UV-B signalling pathways must exist in higher plants (Kalbin et al., 1997, Mackerness et al., 2001, Jenkins et al., 2001, Brosche and Strid, 2003).

1.3.3.5.4 Protein Phosphorylation

For some time, strong evidence has been accumulating that serine / threonine kinases and protein phosphorylation play a key role in UV-B mediated plant signal transduction and *CHS* expression in particular (Harter et al., 1994, Christie and Jenkins, 1996, Frohnmeyer et al., 1997, Brosche and Strid, 2003). Recently, an *Arabidopsis* mitogen-activated protein kinase phosphatase (AtMKP1) has been shown to be required for resistance to UV-C irradiation (Ulm et al., 2001). In addition, studies in tomato cell cultures have demonstrated the induction of a mitogen-activated protein kinase activity, perhaps specifically, by UV-B (Holley et al., 2003).

1.3.3.5.5 Jasmonic Acid, Salicylic Acid and Ethylene

Different pathways involving jasmonic acid, salicylic acid or ethylene, all of which function in wound and / or defence signalling, mediate UV-B induced expression of several genes in *Arabidopsis* (Surplus et al., 1998, Mackerness et al., 1999, Jenkins et al., 2001). Mutant studies have shown that jasmonic acid is involved in UV-B induced expression of protease inhibitor I and II in tomato (Conconi et al., 1996) and *PDF1.2* in *Arabidopsis* (Mackerness et al., 1999). UV-B induced increases in salicylic acid levels apparently mediate *PR-1*, *PR-2* and *PR-5* upregulation in *Arabidopsis* (Surplus et al., 1998), and UV-B induced ethylene increases appear to be involved in upregulating *PR-1* and *PDF1.2* in *Arabidopsis* (Mackerness et al., 1999).

1.3.3.5.6 Nitric Oxide

The use of pharmacological and inhibitor studies to delineate plant signalling mechanisms can be informative but can also frequently prove inconclusive. When an agonist or antagonist fails to have an effect on a system, it is frequently possible to argue that the lack of effect is due to a lack of reagent penetration at the target site in a plant cell. Conversely, when a reagent does appear to induce a significant alteration in response, it is often difficult to demonstrate unequivocally that the effect is a specific and direct consequence of reagent action. In

Arabidopsis conflicting results have been gathered on the role of nitric oxide (NO) in UV-B signal transduction. Nitric oxide synthase (NOS) activity has been measured in plants but whilst nitric oxide scavengers and NOS inhibitors did implicate NO in supplementary UV-B induced regulation of *CHS* in *Arabidopsis* plants (Mackerness et al., 2001, Brosche and Strid, 2003), studies with the same reagents (together with additional work using NO generators) uncovered no evidence for the involvement of NO in *CHS* expression by *Arabidopsis* cells (C. M. Graham and G. I. Jenkins, unpublished work). Clearly, further work is required to resolve conflicting implications.

1.3.3.5.7 ULI3

The *Arabidopsis uli3* (UV-B light insensitive) mutant fails to show normal inhibition of hypocotyl elongation in response to UV-B light (Suesslin and Frohnmeyer, 2003). The mutant also appears to be impaired in other UV-B responses, including the expression of genes such as *CHS*, *PR-1* and *NDPK1a*, suggesting that ULI3 could be involved in UV-B photoreception or early signal transduction. It is worth noting, however, that the *uli3* seedling expression experiments investigated responses to UV-A enriched UV-B light, rather than UV-B specifically. Additionally, *CHS* expression was found also to be reduced in response to UV-A in the *uli3* mutant; so it is conceivable that the wild-type, UV-B induced *CHS* response examined during the characterization of the *uli3* mutant is, in part, dependent on UV-A. The *ULI3* gene (identified by insertional inactivation with Transfer DNA) encodes an 80-kDa protein with potential domains for heme- and diacylglycerol-binding but how it might function in UV-B signal transduction is not clear, although a plausible role for ULI3 in mediating UV-B stimulated electron transfer may be supported by a previous study (Long and Jenkins, 1998, Suesslin and Frohnmeyer, 2003).

1.3.3.5.8 AtMYB4

The *Arabidopsis* R2R3 MYB transcription factor AtMYB4 (Hemm et al., 2001), an orthologue of *Antirrhinum* MYB308 (Jin et al., 2000, Frohnmeyer and Staiger, 2003), appears to regulate phenylpropanoid biosynthesis by repressing transcription of the enzyme cinnamate 4-hydroxylase (C4H). UV-B downregulates the expression of *AtMYB4*, more effectively than either UV-A or UV-C, and this leads to greater expression of *C4H*, allowing greater levels of sinapic acid esters to accumulate in *Arabidopsis* leaves. The result is a plant which displays a greater ability to tolerate the damaging effects of UV-B irradiation. However, AtMYB4 may not have as great an effect on *CHS* expression or flavonoid levels (Brosche and Strid, 2003, Frohnmeyer and Staiger, 2003).

1.3.3.5.9 UVR8

The *Arabidopsis uvr8* mutant was isolated on the basis of its increased sensitivity to supplementary UV-B induced leaf damage (Kliebenstein et al., 2002). The mutant additionally shows other changes in gene expression in response to UV-B, including significantly reduced expression of *CHS* and flavonoids and increased production of PR-1 and PR-5 proteins. However, the mutant plant did not appear to be altered in the expression of the *UVR8* transcript itself nor were alterations apparent in the activities of endogenous antioxidants. It is significant, in the context of the present work, that the UV-B specificity of UVR8 mediated responses was not investigated by Kliebenstein et al.

A single recessive mutation in an *Arabidopsis* gene encoding a predicted protein which is very similar (approximately 35% identical) to the human guanine nucleotide exchange factor Regulator of Chromatin Condensation 1 (RCC1) was found to be responsible for the *uvr8* mutant phenotype (Kliebenstein et al., 2002). The RCC1 family of proteins are nuclear-localized exchange factors for the small G-protein Ran, which is an important component of many nucleocytoplasmic transport pathways. However, the mechanism by which UVR8 mediates *CHS* expression in response to UV-B in *Arabidopsis* has yet to be uncovered and

may well be different from the mechanism by which the corresponding human homologue operates.

The discovery of UVR8, when taken together with the work done on AtMYB4, also shows clearly that different enzymes involved in phenylpropanoid biosynthesis can be controlled by different mechanisms involving both positive and negative regulatory elements (Kliebenstein et al., 2002).

1.3.3.5.10 Other Putative UV-B Signalling Components

In addition to those second messengers and signalling components mentioned above which have been linked to transduction of a UV-B light signal in plants, there are others which are thought to be involved more generally in light signalling pathways. For example, Long and Jenkins provided evidence, based largely on work with inhibitors, that electron transport at the plasma membrane may play a role in both the cry1 and UV-B induction of *CHS* gene expression in *Arabidopsis* (Long and Jenkins, 1998, Jenkins et al., 2001). A number of these more general signalling components also play a crucial role in UV-B signal transduction as discussed below.

1.4 Signal Transduction Components and Second Messengers Generally Involved in Light Signalling

1.4.1 Introduction

The regulation of light responses in *Arabidopsis* depends not only on the perception of different light qualities by the plant, but also upon the manner in which the signals are transduced *en route* to promoting gene expression. Thus the process of signal transduction itself provides an additional layer of complexity which must be taken into account when studying plant responses to light. Numerous illustrations could be given to highlight both variety and complexity involved in the transduction of perceived light signals in plants such as *Arabidopsis*. Photoreceptors, such as PHYB and CRY2, can interact directly (Mas et al., 2000), pathways can remain separate or converge downstream producing additive or synergistic effects (Fuglevand et al., 1996). Some signal transduction components act as control points between photoreceptor systems (Neff et al., 1999), some exhibit varying degrees of redundancy, and universal regulatory elements such as calcium, inositol phospholipids, protein kinases and phosphatases have different functions in different situations (Baum et al., 1999, Wang, 2000, Satterlee and Sussman, 1998, Chrispeels et al., 1999).

1.4.2 The COP/DET/FUS Proteins

Dark grown mutant plants which resemble light-grown seedlings, have proved fruitful in the identification of a series of nuclear-localized negative regulators. The COP / DET / FUS repressors act downstream of several photoreceptors (Whitelam and Devlin, 1998, Walters et al., 1999), and may prevent photomorphogenesis in dark grown plants by mediating specific protein degradation (Schwechheimer and Deng, 2000). Mutations in *Arabidopsis DET1* are epistatic to those in the phytochrome genes (Mustilli et al., 1999), *DET2* encodes a steroid 5 alpha-reductase (Noguchi et al., 1999) and DET3 corresponds to subunit C of the vacuolar H+-ATPase (Schumacher et al., 1999). Studies by Kim *et. al.* have suggested that both the

COP1 and DET1 proteins are involved in UV-B induced inhibition of *Arabidopsis* hypocotyl elongation (Kim et al., 1998).

1.4.3 HY5

HY5 is a basic leucine zipper (bZIP) transcription factor which binds directly (Osterlund et al., 2000) to the promoters of light-inducible genes promoting photomorphogenesis – the phyto-developmental mode which is specific to growth under light. *Arabidopsis* HY5 is also required for UV-A / blue and UV-B induced *CHS* expression in mature leaf tissue (Wade, 1999) and the nuclear abundance of this transcription factor is enhanced after illumination (Hardtke et al., 2000). Added stability is conferred on HY5 by a specific kinase activity, probably that of casein kinase II, which phosphorylates the protein in its COP1 binding domain rendering HY5 less susceptible to COP1 mediated degradation - but less active in binding target promoters. A small pool of less reactive, phosphorylated HY5 may be maintained in the dark to effect a rapid transition to photomorphogenesis when a plant is first illuminated (Osterlund et al., 2000, Hardtke et al., 2000).

1.4.4 COP1

Arabidopsis COP1 is a RING-finger / WD-40 repeat protein which interacts, as a homodimer, directly with HY5. Significantly, COP1 nuclear abundance is down-regulated by light. The result of the COP1-HY5 interaction is the targeted degradation of unphosphorylated HY5 by the 26S proteasome and repression of the photomorphogenic developmental program in dark grown plants (Holm et al., 2001, Stacey et al., 2000, Osterlund et al., 2000, Stoop-Myer et al., 1999, Osterlund et al., 1999, Holm and Deng, 1999, Torii et al., 1998). The 26S proteasome, which breaks down intracellular proteins targeted by ubiquitin conjugation, is also involved in modulating PHYA levels (Clough and Vierstra, 1997, Clough et al., 1999, Fu et al., 1999a, Fu et al., 1999b). A number of proteins which interact with COP1 have been identified (CIP1, CIP7 and CIP8), but the mechanics of their regulatory effect is not altogether clear (Wei and Deng, 1996, Torii and Deng, 1997, Yamamoto et al., 1998, Torii et al., 1999). The key events,

in the light grown plant, appear to be: COP1 is excluded from nuclei so HY5 accumulates and binds to light-regulated gene promoters, promoting photomorphogenesis (Schwechheimer and Deng, 2000).

1.4.5 COP9

The COP9 signalosome (CSN) is an eight-subunit protein complex which regulates ubiquitination and protein turnover in plant developmental and physiological processes, including photomorphogenesis, hormone signalling, and defense against pathogens (von Arnim, 2003). The CSN appears to repress photomorphogenesis in *Arabidopsis* by regulating the nuclear abundance of COP1 (Yahalom et al., 2001, Mundt et al., 1999).

1.4.6 Cross Talk and Synergism

With such a variety of light qualities and quantities incident, it is crucial that *Arabidopsis* follows the most appropriate developmental response in a given situation. It appears that plant signal transduction pathways can exchange information and integrate inputs from more than one stimulus (Moller and Chua, 1999, Genoud and Metraux, 1999). Presumably, such cross-talk has arisen because of its adaptive value and the elucidation of the interacting pathways may provide insights into how natural selection operates at the molecular level. For example, PHYA and PHYB can act antagonistically or synergistically in the control of particular responses. Similarly, CRY1 and PHYB can act in a synergistic or additive fashion depending upon the light regime. Phototropism in unilateral blue light appears to be mediated primarily by the phototropins, but there is evidence that CRY1, CRY2, PHYA and PHYB regulate this response too (Casal, 2000, Ballare and Casal, 2000), and the recently identified cryptochrome signalling component SUB1, a Ca^{2+}-binding protein that suppresses light-dependent accumulation of HY5, also functions as a modulator of a phytochrome pathway (Guo et al., 2001).

1.4.7 MYB Transcription Factors

Recent work on the role of plant regulators underpinning tolerance of ultraviolet light has focused on MYB transcription factors. Different categories of this large class of proteins can be identified by the number of DNA-binding MYB domain repeats. Plant MYBs have a variety of roles including the regulation of secondary metabolism, meristem formation, the cell cycle and cellular morphogenesis. As discussed above, it now appears that the *Arabidopsis thaliana* MYB, AtMYB4, is a key regulator of phenylpropanoid pathway gene expression and the first example of a MYB transcriptional repressor (Jin and Martin, 1999, Romero et al., 1998, Jin et al., 2000, de Majnik et al., 2000).

In addition, it has been demonstrated that MYB75 and MYB90 are involved in upregulating flavonoid biosynthetic gene expression in *Arabidopsis*, with MYB75 playing a definite role in *CHS* expression (Borevitz et al., 2000).

1.4.8 *cis* Elements of the *Arabidopsis CHS* Gene

Arabidopsis CHS promoter constructions were used in conjunction with a transient expression system developed in protoplasts to identify a −164 bp *Arabidopsis CHS* light-responsive unit (LRU). This LRU^{AtCHS} has an ACGT containing element (ACE) and a MYB recognition element (MRE), similar to those found in the parsley *CHS* promoter (Hartmann et al., 1998), which play a key role in conferring UV-B and UV-A / blue light mediated *CHS* expression. Since studies in parsley have shown that the ACE^{PcCHS} and MRE^{PcCHS} elements bind bZIP and MYB type transcription factors respectively (Feldbrugge et al., 1994, Feldbrugge et al., 1997), it is tempting to speculate that the corresponding elements in *Arabidopsis* may bind homologous transcription factors such as HY5 (bZIP), MYB75 and / or possibly MYB90 (Figure 1.7) – although it should be noted that the ACGT element appears to be capable of binding bHLH factors in addition to bZIPs. Further sequences upstream of the LRU^{AtCHS} were also found to contribute to light induced *CHS* expression in *Arabidopsis*. It therefore seems likely that although the UV-B and UV-A / blue phototransduction pathways are distinct in *Arabidopsis* (Christie and Jenkins, 1996, Fuglevand et al., 1996), the signalling routes

converge on similar transcription factor(s) and the same promoter elements to induce *CHS* expression. Additionally, some factor(s) appear to be synthesized *de novo* in response to illumination because UV-B and UV-A / blue induced *CHS* expression in both parsley (Feldbrugge et al., 1996) and *Arabidopsis* (Christie and Jenkins, 1996) is blocked by cycloheximide – an inhibitor of eukaryotic protein synthesis (Hartmann et al., 1998).

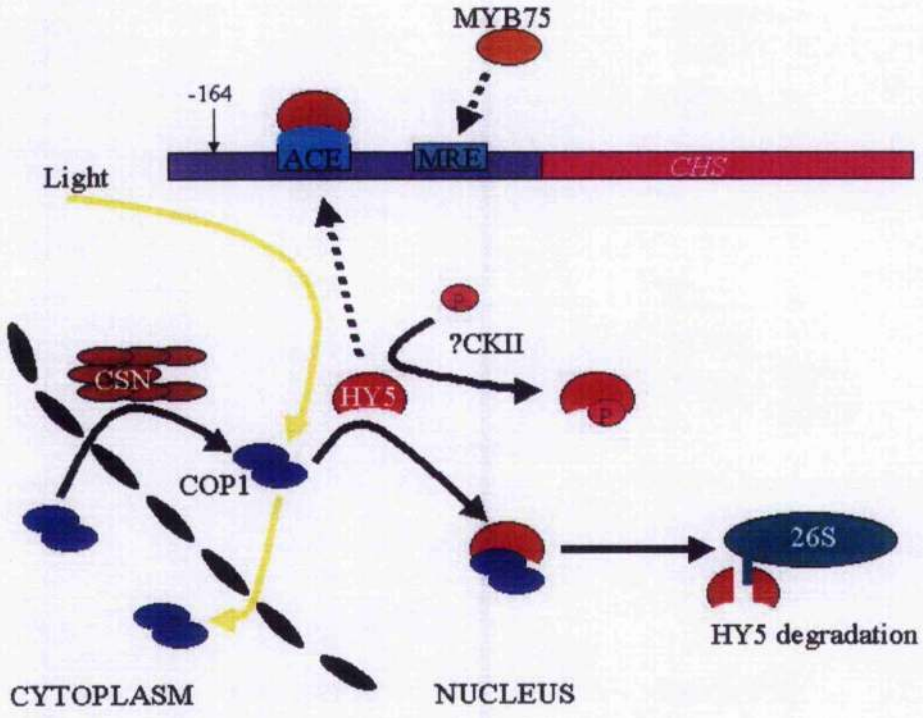


Figure 1.7
Proposed model for downstream regulation of *CHS* expression in *Arabidopsis*.
CKII, casein kinase II; CSN, COP9 signalosome; 26S, 26S proteasome; ACE, ACGT containing element; MRE, MYB recognition element; Dashed lines represent possible DNA – protein interactions (Hardtke et al., 2000, Hardtke and Deng, 2000, Borevitz et al., 2000, Hardtke et al., 2002).

1.5 The Phenylpropanoid Pathway and Flavonoid Biosynthesis

1.5.1 Introduction

One of the major effects ultraviolet and blue light has on *Arabidopsis thaliana* is to increase the levels of secondary metabolites which protect the plant against damaging short-wavelength radiation. The phenylpropanoid pathway (Figure 1.8) produces thousands of compounds, including flavonoids such as flavonols, anthocyanidins and tannins (Schoenbohm et al., 2000, Weisshaar and Jenkins, 1998). Phenylpropanoids play a variety of defensive roles in plants; these include UV protection and antioxidation and assume particular importance in attenuating the damaging effects of high-energy light on DNA and other biomolecules (Mackerness, 2000, Landry et al., 1995, Mazza et al., 2000). Complex metabolic pathways (eg. the shikimate pathway) often require the co-ordinate expression of a series of enzymes in order to function optimally, and recent work suggests that phenylpropanoid and flavonoid metabolism is catalyzed by one or more membrane-associated multienzyme complexes (Winkel-Shirley, 1999, Burbulis and Winkel-Shirley, 1999).

1.5.2 Evolution

Despite the diversity of products in plant secondary metabolism the number of different types of reaction is actually quite small; the explanation for this appears to be repeated evolution (a special form of convergence) and the implication for researchers identifying associated genes is that assignment of function based on sequence information alone should be undertaken with caution (Pichersky and Gang, 2000). Most enzymes involved in plant secondary metabolism are encoded by small families of genes which originated by duplication. In *Ipomoea*, multiple copies of the enzyme which catalyzes the first committed step in flavonoid biosynthesis, chalcone synthase, illustrate gene duplication, whilst in *Arabidopsis* there is only one *CHS* gene. Evolution is often accelerated in newly duplicated genes, and evidence exists for shifts in enzymatic function. Thus, researchers should note that recurrent gene duplication and

diversification appears to be a common adaptive strategy in plant genome evolution (Durbin et al., 2000, Blanc et al., 2000, Li, 1997).

1.5.3 Phenylalanine Ammonia-lyase (PAL) and Chalcone Synthase (CHS)

The first step of the phenylpropanoid pathway is catalyzed by phenylalanine ammonia-lyase (PAL) which in *Arabidopsis*, as in many species, is encoded by a multi-gene family (Wanner et al., 1995). By contrast, the *Arabidopsis* enzyme chalcone synthase which further commits the phenylpropanoid pathway to flavonoid biosynthesis is encoded by a single gene (Shirley et al., 1995) and the expression of *CHS* is controlled principally at the level of transcription. Both enzyme transcripts accumulate in response to UV-B and UV-A / blue light in *Arabidopsis* cell suspension culture *via* signal transduction pathways which may involve plasma membrane redox activity (Long and Jenkins, 1998). Both enzymes are also induced, in *Arabidopsis* leaves, by low temperature in a light-dependent manner (Leyva et al., 1995) and both are elevated in response to treatment with exogenous cytokinins, reflecting a related increase in anthocyanin levels (Deikman and Hammer, 1995). As PAL and CHS are enzymes which catalyze reactions forming branch-points in secondary metabolism, understanding the control of their expression provides insight into how and why plants respond to different types of stress.

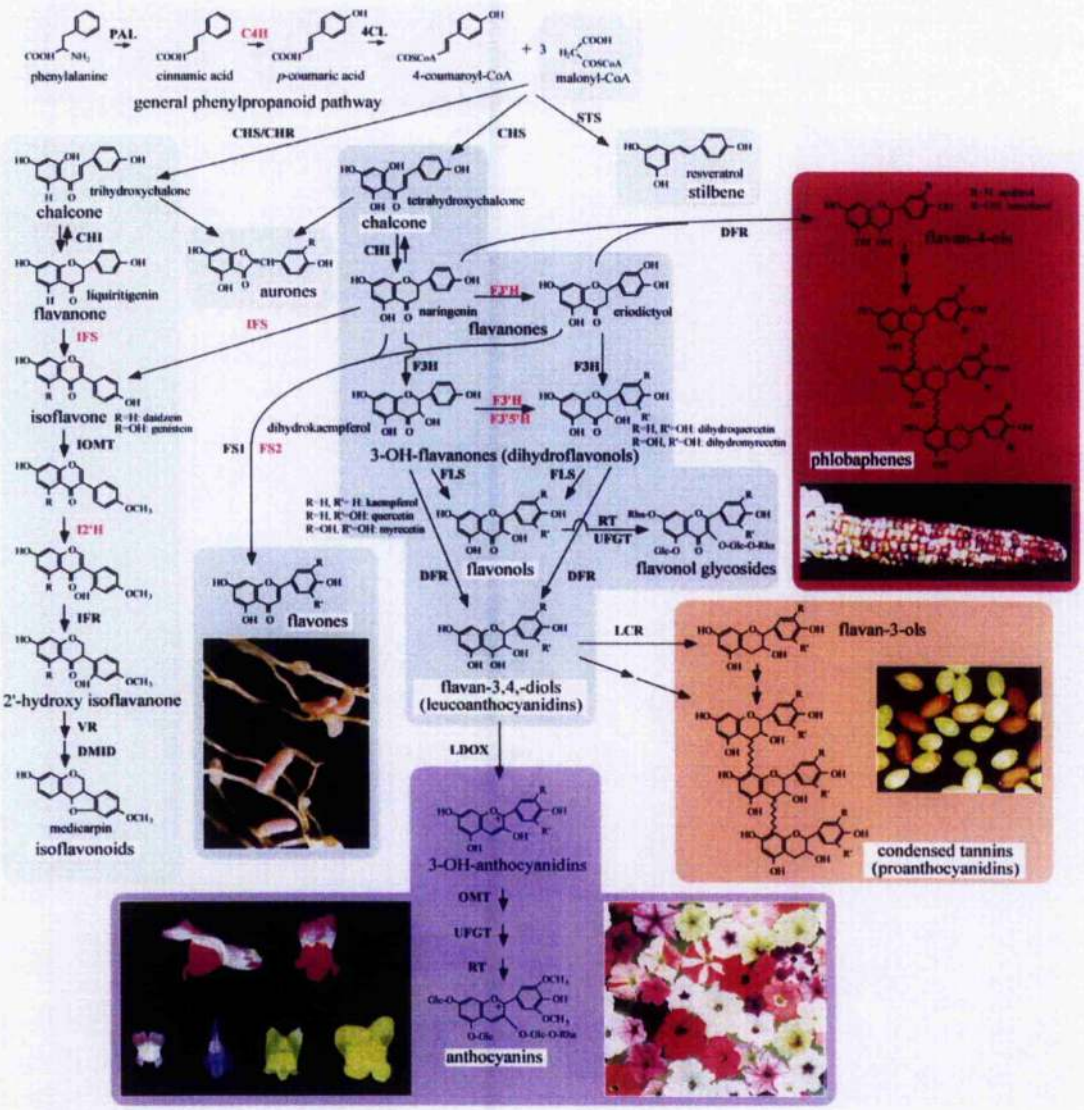


Figure 1.8

Summary of Phenylpropanoid Biosynthesis (Taken from Winkel-Shirley, 2001).

Schematic of the major branch pathways of flavonoid biosynthesis, starting with general phenylpropanoid metabolism and leading to the nine major subgroups: the colourless chalcones, aurones, isoflavonoids, flavones, flavonols, and flavandiols (gray boxes), and the anthocyanins, condensed tannins, and phlobaphene pigments (colored boxes). Enzyme names are abbreviated as follows: cinnamate-4-hydroxylase (C4H), chalcone isomerase (CHI), chalcone reductase (CHR), chalcone synthase (CHS), 4-coumaroyl:CoA-ligase (4CL), dihydroflavonol 4-reductase (DFR), 7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase (DMID), flavanone 3-hydroxylase (F3H), flavone synthase (FSI and FSII), flavonoid 3' hydroxylase (F3'H) or flavonoid 3'5' hydroxylase (F3'5'H), isoflavone *O*-methyltransferase (IOMT), isoflavone reductase (IFR), isoflavone 2'-hydroxylase (I2'H), isoflavone synthase (IFS), leucoanthocyanidin dioxygenase (LDOX), leucoanthocyanidin reductase (LCR), *O*-methyltransferase (OMT), Phe ammonia-lyase (PAL), rhamnosyl transferase (RT), stilbene synthase (STS), UDPG-flavonoid glucosyl transferase (UFGT), and vestitone reductase (VR). Photographs are courtesy of Cathie Martin (John Innes Centre, Norwich, UK; *Antirrhinum*), Francesca Quattrocchio (Free University, Amsterdam; petunia), Erich Grotewold (Ohio State University, Columbus; maize), and Yimei Lin and Ann Hirsch (University of California, Los Angeles; sweet clover).

1.6 A Genetic Approach to Understanding Light Responses in *Arabidopsis*

1.6.1 Introduction

The small crucifer *Arabidopsis thaliana* has been the focus of plant molecular genetic studies for over twenty years and owes its model organism status to its small size, short generation time, fecundity, small genome and the ease with which *Arabidopsis* mutants can be isolated (Meinke et al., 1998). The *Arabidopsis* genome was sequenced by the end of the year 2000 and comprises five homologous pairs of chromosomes and approximately 157 Mb DNA (Bennett et al., 2003, Bennett and Leitch, 2005). Hard on the heels of this landmark discovery in higher plants came another milestone: a draft completion of the 3,200 Mb (23 chromosome pairs) human genome sequence early in 2001. If the initial analyses are reliable then the difficult work in plant biology has barely begun, for *Arabidopsis* has around 26,000 genes - a figure similar to that estimated (30,000 genes) for *Homo* (http://www.nature.ca/genome/03/a/03a_11a_e.cfm). Remarkably, at least 70% of the *Arabidopsis* genome has been duplicated and there are fewer than 15,000 different genes (Venter et al., 2001, Walbot, 2000). The extensive similarities between those genomes sequenced so far, and the high level of genetic redundancy apparent within genomes means that researchers face a significant challenge to try to understand what lies at the root of complexity in living things and what makes plants, animals and microbes so very different as organisms (Lagercrantz, 1998, Powledge, 2000, Walbot, 2000). It seems likely that the biological causes of distinctiveness are at least as much a product of the way in which essentially the same gene products interact in different organisms, as they are the sum of differences in the gene products themselves.

1.6.2 Mutant Studies

One of the best ways to understand how a system works can be to remove a component and observe the result. If a component is lost, modified or damaged then the system may remain functional but changed in a way that reveals the significance of the altered component. The

biological equivalent of removing a component from a mechanical system is called 'the genetic approach' to understanding gene function, and the cornerstone of this approach is mutant studies. *Arabidopsis* mutants can be generated using agents such as ethyl methane sulfonate (EMS) and x-rays, or by the introduction of Transfer DNA (T-DNA) or transposable elements (transposons) (Russell, 1998, Li et al., 1999). Since *Arabidopsis* is diploid and a wild-type gene product from a homologous chromosome may mask the presence of a mutant protein, second generation (M_2) plants are screened for recessive mutations. Mutant phenotypes can be characterized by observation, measurement, HPLC, physiological and pharmacological studies, whilst gene expression can be quantified by northern blot or RT-PCR. For example, in *Arabidopsis* the onset of leaf hair (trichome) development is prevented by mutations in at least two genes - *GL1* and *TTG*. The latter gene is pleiotropic, additionally affecting anthocyanin accumulation, root hair development and seed coat mucilage production (Larkin et al., 1999, Lackie and Dow, 1999).

1.6.3 Epistasis

By a simple cross-breeding procedure and subsequent selection, it is possible to generate 'double-mutants' which are deficient in two non-allelic genes. One can then investigate whether the altered genes have additive effects on a phenotype. Double mutants can also be used to determine which of two gene products operating in the same signalling pathway acts earlier. A gene which masks the expression of a non-allelic gene is said to be epistatic to the gene with the obscured effect (Russell, 1998). For example, double mutant studies on the *Arabidopsis FUSCA* genes have shown that these are epistatic to the phytochrome deficient *hy* mutants, and consequently act downstream of phytochrome. However, mutations in *TTG* suppress anthocyanin build-up in *fusca* double mutants, indicating that the FUSCA gene product(s) act upstream of TTG (Misera et al., 1994).

1.6.4 Map-based Cloning

Recent advances in *Arabidopsis* molecular genetics have meant that facilities available for the isolation of genes by insertional inactivation (tagging) or map-based cloning have never been better. There are many T-DNA tagged and transposon seed lines, detailed physical maps highlight numerous polymorphic locii and the complete *Arabidopsis* genomic sequence is available for both the Landsberg *erecta* and Columbia ecotypes (Jander et al., 2002, Li et al., 1999, Lukowitz et al., 2000, Feldmann, 1991). Even so, map-based cloning can be a demanding and time-consuming exercise and there are a number of potential pitfalls. Once a candidate gene has been identified, a wild-type copy can be re-introduced to the mutant plant using *Agrobacterium tumefaciens* (Clough and Bent, 1998, Liu et al., 1999). Complementation of the mutant is confirmed if the wild-type phenotype is restored, and the corresponding gene can then be sequenced (to identify regions of functional importance which may be altered in the mutant allele), overexpressed or compared to genes of similar sequence and known function to try to understand its role *in vivo* (Dean and Schmidt, 1995, ArnholdtSchmitt, 1996).

1.6.5 The Limitations of the Genetic Approach

Not every gene involved in a particular plant physiological or developmental process will be amenable to investigation by the genetic approach. In those cases where a mutated gene is redundant because another shares the same function, the plant will not display a mutant phenotype unless the second gene has also been mutated – and this is clearly vanishingly unlikely.

For the purposes of identifying mutant genes, having a T-DNA or transposon derived mutant is a significant advantage because a large insert with a clearly defined DNA sequence should not be difficult to find in the genome. However, mutagens which generally produce much smaller changes, such as EMS, tend to produce a greater variety of mutations and can lead to the discovery of genes which would not be found by the more deleterious gene tagging procedures. To locate mutant genes generated using EMS however, it is often necessary to

resort to map-based cloning and although this procedure has become much easier in recent years, there is still a number of potential difficulties which could be encountered.

It is certainly a big advantage, for map-based cloning, to have a mutant with a clear, visible phenotype which segregates in a Mendelian fashion. Having a mutant genotype which completely penetrates the phenotype only under certain conditions certainly makes identification of a mutant gene more difficult. Other complications which can frustrate an attempt to clone a gene based on its map position include situations where a phenotype is caused by multiple mutations, or by epigenetic or even non-nuclear mutations (Jander et al., 2002, Lukowitz et al., 2000).

Designing a good screen for deficiencies of a particular phenotype is the first step, and one of critical importance, in applying the genetic approach. For example, *Arabidopsis* mutants which show an increased susceptibility to leaf damage and impaired growth in response to high doses of UV-B light, such as *uvr2-1* (impaired in CPD photolyase gene *PHR1*) and *uvr3* (deficient in 6-4 PP photolyase gene and corresponding photoreactivation), may be defective in DNA repair or phenolic sunscreen biosynthesis whilst being entirely unaltered in their ability to perceive and transduce the UV-B signal (Landry et al., 1997, Nakajima et al., 1998, Frohnmeyer and Staiger, 2003). Screening for mutants altered in the UV-B signalling machinery may require low doses of non-supplementary UV-B which are sufficient to induce photomorphogenesis but insufficient to cause DNA damage (Ballare et al., 1991, Kim et al., 1998, Suesslin and Frohnmeyer, 2003).

Once a practical screen has been developed which allows the investigator to look through vast quantities of plants for clearly defined mutations, populations of mutagenized M_2 seeds are usually generated for screening. Putative mutants are isolated from the screen and characterized to understand more about how the corresponding mutations impact upon the plant. Existing mutants with similar phenotypes are compared and perhaps cross-pollinated with the new mutants to see if the generated mutations are truly novel or alleles of existing mutant genes. If the mutations appear to be novel, attempts may be made to clone and identify the altered genes.

1.7 The Objectives of this Study

The focus of the studies reported herein was to try to understand how UV-B light elicits responses in *Arabidopsis thaliana*. A genetic approach was taken because the isolation of mutants has been a key step in some of the major advances in our comprehension of plant photoperception and signal transduction over the last 20 years. The first step in our genetic approach to understanding the responses of *Arabidopsis* to UV-B was to identify clear responses to UV-B which could form bases for mutant screens (Chapter Three). Several responses to UV-B were examined and screens developed to isolate mutants altered in UV-B induced *CHS* gene expression (Chapter Four) and UV-B induced phototropism (Chapter Five). A screen for mutants altered in UV-B induced inhibition of hypocotyl elongation was found to be impractical, whilst a screen for mutants with an increased susceptibility to supplementary UV-B induced leaf tissue damage and growth inhibition may yet be followed up. The search for mutants altered in *CHS* gene expression proved to be particularly fruitful, resulting in the characterization of a mutant (designated *chum31*) deficient specifically in responses to UV-B (Chapter Four), as distinct from generally altered in UV-A and UV-B responses. The *chum31* mutant gene was shown to be an allele of *UVR8* (Kliebenstein et al., 2002) and the corresponding protein implicated in the regulation of other phenylpropanoid biosynthetic enzymes and, significantly, the transcription factor HY5.

Additionally, a lengthy map-based cloning project was undertaken (Chapter Six) to identify the gene responsible for the chlorotic and reticulated visible phenotype observed in a mutant called *30e5* which was previously isolated in our lab (Fuglevand et al., unpublished work). The *30e5* mutant was shown to have increased levels of leaf tissue damage and growth inhibition in response to supplementary UV-B light.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The chemicals used in this study were obtained from VWR International Ltd. (Lutterworth, Leicestershire), Sigma (Poole, Dorset) and Fisher Scientific (Loughborough, Leicestershire). Specialist chemicals and reagents were supplied as indicated.

2.1.2 DNA Modifying Enzymes

DNA restriction, synthesis and modification enzymes were purchased from Promega (Southampton, U.K.), New England Biolabs (Hitchin, Hertfordshire), Invitrogen (Paisley, Scotland), Roche Diagnostics (Mannheim, Germany) or as indicated. Enzymes were used in accordance with the manufacturer's instructions.

2.2 General Laboratory Procedures

2.2.1 pH Measurement

The pH of solutions was measured using a Jenway 3320 pH meter and glass electrode (Jenway, Felsted, Essex).

2.2.2 Autoclave Sterilization

Equipment and solutions were sterilized at 15 psi for approximately 30 minutes using benchtop (eg. Prestige Medical, Model 220140) or free-standing (eg. Laboratory Thermal Equipment Autoclave 225E) autoclaves.

2.2.3 Filter Sterilization

Heat labile solutions were sterilized by filtration through a Nalgene filter (pore diameter 0.2 μM) and collected in a sterile container.

2.2.4 Solutions and Equipment for RNA Work

Solutions for RNA work were treated with 0.05 % (v/v) diethyl pyrocarbonate (DEPC; Sigma) overnight to denature ribonucleases (RNAses) present, before the DEPC was destroyed by autoclaving. Sterilized plasticware was used throughout.

2.2.5 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction was carried out using DYAD DNA Engine PTC-220 and DNA Engine PTC-200 Peltier Thermal Cycler (Genetic Research Instrumentation, Braintree, Essex, U.K.) machines.

2.3 Plant Material

2.3.1 Seed Stocks

Wild-type *Arabidopsis thaliana* cv. Landsberg *erecta* and Col3 seeds were obtained from The European *Arabidopsis* Stock Centre (ie. NASC, Nottingham, UK). Also provided by NASC in the L. *er* ecotype were the *tt4*, *tt5*, *cry1* (*hy4-2.23N*), *cry2* (*fha1*) and *hy5-1* mutants. NASC also supplied, in the Columbia background, *fah1-2* and *fah1-7* together with a number of SALK T-DNA insertion lines (see Chapter Six). The *fl196* (N458) and *dov1* variegated mutants were provided in the En-2 ecotype, also by NASC. The *icx1* (Jackson et al., 1995) and *30e5* (Fuglevand and Jenkins, unpublished work) mutants were both isolated from a L. *er* background here in Glasgow. The *CHS-Luc* lines (L. *er*) were also generated (by Dr. Matthew Shenton) and characterized (by George Littlejohn) in Professor Jenkins' Glasgow University Lab. Dr. Dan Kliebenstein (University of California, Davis, U.S.A.) provided the *uvr8-1* mutant (L. *er*) which has been of inestimable value. The *phot1-5* and *phot2-1* single, together with the *phot1-5phot2-1* double mutants were kindly donated by Dr. John Christie (University of Glasgow) and Professor Winslow Briggs (Carnegie Institution of Washington, Stanford, U.S.A.); these lines were all generated in a Columbia background, as was the *cry2-1cry1-304* double mutant from Professor Chentao Lin (University of California, Los Angeles, U.S.A). The *atd2* mutant was provided by Professor Ralf Boldt (University of Rostock, Rostock, Germany) in the C24 background and the *phot1cry1cry2* and *cry1cry2hy1* triple mutants (each in a mixed ecotype) were gifts of Dr. Enrique Lopez-Juez (Royal Holloway, University of London, Surrey, U.K.). The *phyA-1phyB-1* mutant (L. *er*) was from Professor Garry Whitelam (University of Leicester, Leicester, U.K.).

2.3.2 Growth and Harvesting of Compost Grown Plants

Arabidopsis seeds were sown on the surface of pots containing autoclaved (1 hour at 15 psi) compost (Growers Potting and Bedding Compost, William Sinclair Horticulture Ltd., Lincoln, U.K.) soaked in 0.15 g l^{-1} of a solution of the insecticide Intercept® (Scotts U.K., Bramford, Ipswich). Intercept® has been shown to be highly effective against aphids. The pots were then covered with cling film and vernalized for 2-4 days at 4 °C before transfer to growth conditions at 20 °C. Cling film was generally retained for the first week of growth to maintain humidity at this crucial stage of development and overcrowding was alleviated prior to light treatments by discarding excess plants. The insecticide Conserve® (Fargro Ltd., Littlehampton, West Sussex) was used to control thrips. Seedlings were grown for the durations and at the fluence rates described (eg. 21 days at 25 $\mu Em^{-2}s^{-1}$ white light; stated throughout as 'low white light') before the treatments specified were applied. After treatments, harvested leaf tissue samples were packaged into aluminium foil and frozen directly into liquid nitrogen before storage at –80 °C. Alternatively, treated plants were photographed, imaged, stained or manipulated as described. Addition of thiamine to the *30e5* mutant grown on compost (described in Section 6.2) was effected by watering plants regularly with a tap-water solution of 1 % (w/v) thiamine.

2.3.3 Seed Surface Sterilization Procedures

In order to grow *Arabidopsis* on agar plates, seed surface sterilization was generally carried out using one of the following two procedures:

2.3.3.1 Seed Surface Sterilization Method One

Arabidopsis seeds were placed on a Whatman (9 cm diameter) filter paper circle which was then folded into quarters and the seeds secured inside using a paper clip to seal the upper flap. The packet was immersed in 70 % (v/v) ethanol inside a Magenta jar for 2 minutes and drained, before the addition of 10 % sodium hypochlorite (≥1.2 % (w/v) available chlorine), 0.02 % (v/v) Triton X-100 for 10 minutes with occasional agitation. Further manipulations

were carried out inside a sterile flow cabinet. Using sterilized forceps, each packet of seed was rinsed five times in a fresh Magenta jar containing sterile dH_2O. The packets were then allowed to dry for several hours on a sterile-surface (eg. a Petri dish lid) inside the flow cabinet before the seeds were sown on agar plates using sterilized forceps. Plates containing seeds were sealed with Micropore® tape and vernalized at 4 °C for 2-4 days before transfer to growth conditions.

2.3.3.2 Seed Surface Sterilization Method Two

A small amount of *Arabidopsis* seeds were placed in the bottom of a sealable plastic tube (eg. an Eppendorf®). A 95 % (v/v) solution of ethanol was added for 1 minute and the tube inverted to ensure all the seeds were immersed. The seeds were then pelleted by centrifugation at 15,000 *g* for 1 minute and the ethanol drawn off with a pipette. Next, a solution containing 50 % sodium hypochlorite (≥6 % (w/v) available chlorine), 0.05 % (v/v) Triton X-100 was added for 4 minutes and the tube sealed and inverted several times. The seeds were again pelleted by centrifugation at 15,000 *g* for 1 minute. Further manipulations were carried out inside a sterile flow cabinet using sterile equipment and solutions. The sodium hypochlorite solution was drawn off with a pipette and the seeds rinsed three times with sterile dH_2O, 0.05 % (v/v) Triton X-100. Centrifugation was used to pellet the seeds after each wash and the wash solution removed using a pipette. Finally, the seeds were resuspended in a 0.1 % (w/v) solution of sterile agarose and could be poured or pipetted (using a cut-off sterile pipette tip) onto the surface of an agar plate. Agar plates were sealed with Micropore® tape to allow gas exchange and vernalized at 4 °C for 2-4 days before being transferred to a growth chamber.

2.3.4 Growth and Harvesting of Seedlings Grown on Agar Plates

For growth on agar plates, *Arabidopsis* seeds were surface sterilized and sown as described in Section 2.3.3. Growth media for seedlings consisted of 1 x Murashige and Skoog (MS) Basalt Salts Mixture (Sigma), 0.5 g l^{-1} 2-(N-Morpholino) Ethanesulfonic acid (MES), 100 mg l^{-1}

inositol, 1 mg l^{-1} thiamine, 0.5 mg l^{-1} pyridoxine, 0.5 mg l^{-1} nicotinic acid, 0.8 % (w/v) agar, adjusted to pH 5.7. For media containing sucrose (such as that used in the phototropism screen detailed in Chapter Five), this was added to a concentration of 2 % (w/v). For those experiments designed solely to look at the effect of sucrose on *CHS* expression in the *uvr8* mutants, the media used consisted of 1 x MS Salts, 1 x B5 vitamins, 0.8 % (w/v) agar, adjusted to pH 5.7 and with 2 % (w/v) sucrose added if appropriate, all as per H. K. Wade's method (Wade, 1999). All media was sterilized by autoclaving and poured into Petri dishes in a sterile flow cabinet. For generating etiolated seedlings, plates were wrapped in aluminium foil before vernalization and germinated upright in a dark growth room at 20 °C for 4 days (or 2 days for hypocotyl growth experiments). Seedlings were harvested and packaged into aluminium foil directly after treatments (or as specified) and frozen in liquid nitrogen before storage at –80 °C. Etiolated seedlings were harvested under a safe-light. A brief pulse of light was used, before dark treatment, to synchronize the germination of seedlings screened for mutants deficient in UV-B induced positive phototropic hypocotyl curvature (see Section 2.9.3). Seedlings grown under white light on agar plates were positioned on the bottom shelf of each growth room to minimize the build-up of condensation on Petri dish lids.

2.3.5 Cross-Pollination of *Arabidopsis*

Several pots of each parent plant to be used in crossing were grown up in relatively high fluence rate (eg. 100 $\mu Em^{-2}s^{-1}$) white light. High fluence rates will generate more robust plants which will flower sooner than will those grown under low fluence rates. Staggering the age of a series of identical crossing trays is also valuable, insofar as it maximizes the chances of completing a series of successful crosses (especially if some parent plants develop slowly). Female parents are ready for crossing when they have just begun to bolt, male parents when they are starting to produce flowers. Fine forceps should be used and frequently cleaned with a damp tissue, especially between crosses. Plants were watered before emasculation and all opened flowers and those buds not selected for crossing removed from the female parent to leave just a few large, unopened buds. These remaining unopened buds were carefully

dissected using a Nikon (Tokyo, Japan) stereo-microscope (model 85525). All organs (sepals, petals and stamens) were removed except the pistil. An open flower from the male parent, containing bright yellow pollen, was removed and used to fertilize the stigma of the female parent plant. Each cross was then surrounded with a clear polythene protective film and secured to a support with a label containing a description of the two parents. A propagator lid was used to cover the crosses overnight and then removed (to briefly create a humid environment). The F_1 generation seed was harvested after each silique had elongated and had begun to brown.

2.4 Plant Treatments

2.4.1 Illumination of Plant Material

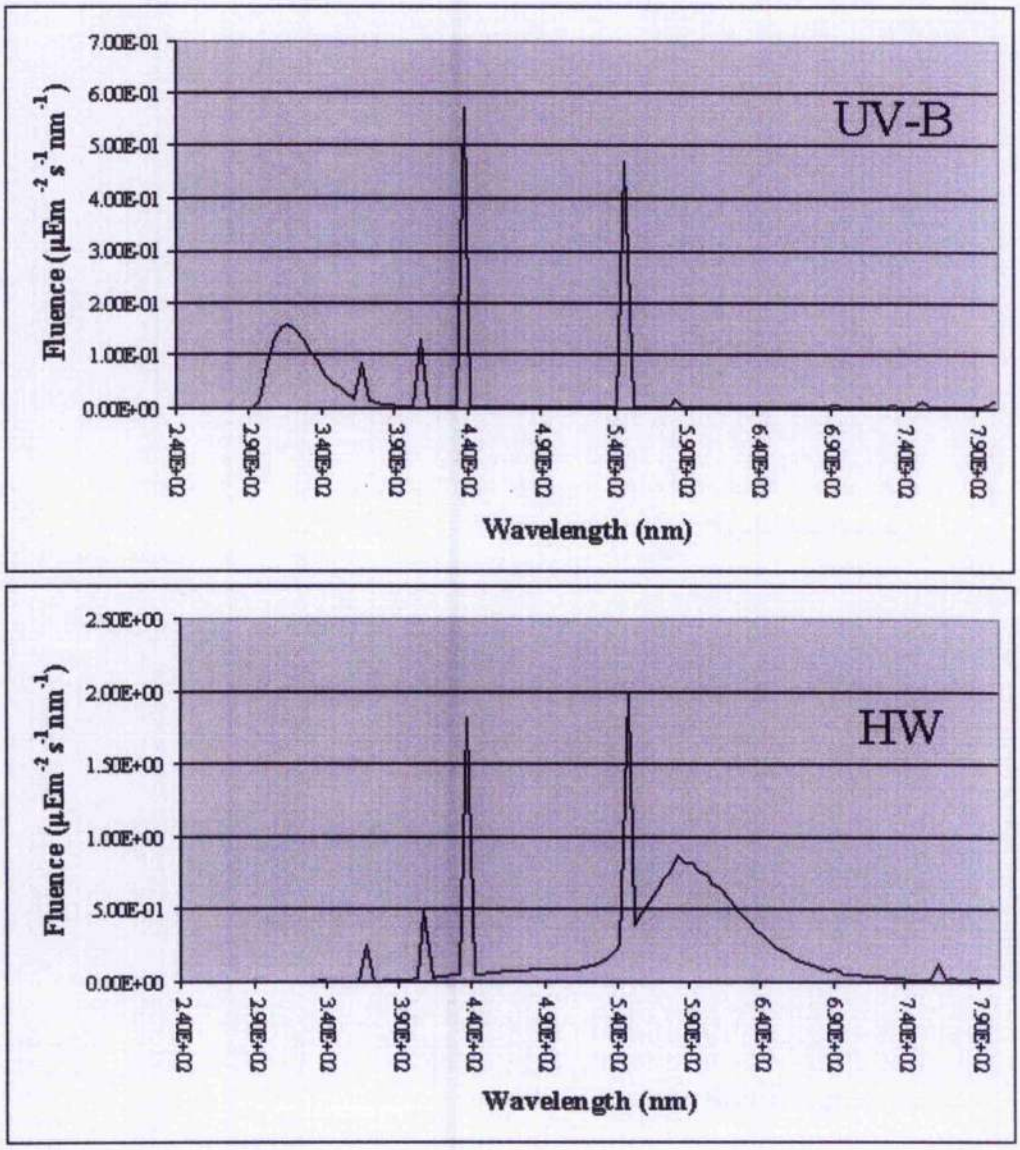
2.4.1.1 Light Sources

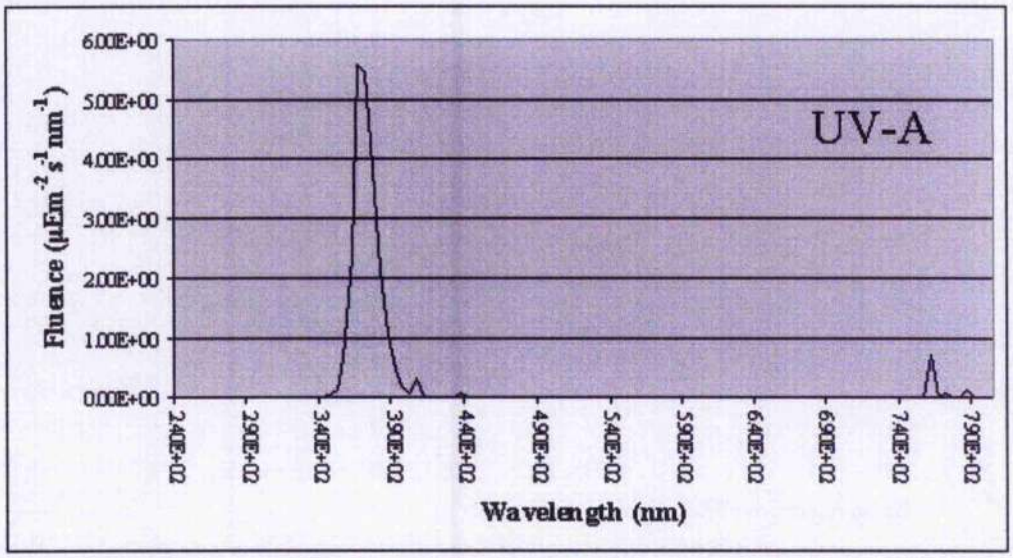
Illuminations were carried out in controlled environment rooms at 20 °C. Spectra of light qualities used were generated using a Macam Spectroradiometer SR9910 (Macam Photometrics Ltd., Livingston, Scotland) and are shown in Figure 2.1. White light was provided by warm white fluorescent tubes eg. L36W/30 (Osram, Munich, Germany). UV-A light was obtained from Sylvania F36W/BLB-T8 blacklight-blue tubes (GTE Sylvania, Shipley, U.K.). UV-B light was derived from Q-Panel UV-B 313 tubes (Q-Panel Co., U.S.A.). A cellulose acetate filter (Catalogue No. FLM400110/2925, West Design Products, Nathan Way, London) was used as standard and changed every 24 hours to eliminate any UV-C (wavelengths <280 nm) from the UV-B tubes (UV-C is therefore absent from the UV-B spectrum in Figure 2.1). A 'Clear 130' filter (Filter No. 130, Lee Filters, Andover, U.K.) was employed in 'UV-B Minus' treatments to ensure *Arabidopsis* responses were specific to UV-B (280-320 nm) wavelengths. Other UV-B sources were additionally tested in preliminary experiments, including Philips TL/01 UV-B fluorescent lamps (Philips, Eindhoven, Netherlands), and monochromatic filters were also examined but these were not used to generate the data shown. Blue light (as shown in Figure 2.1) was generated by covering Osram L36W/67 Blue tubes (Osram, Munich, Germany) with a 'Moonlight Blue' filter (Filter No. 183, Lee Filters, Andover, U.K.) to remove UV-A wavelengths (<390 nm). Far red light was provided by Toshiba FL20S FR-74 tubes (Toshiba, Japan). Fluence rates were adjusted to the quantities described in the text, by varying both the number of fluorescent tubes used in a light treatment and the distance between the tubes and the plant material being illuminated. For the phototropism screen, very low fluence rates of UV-B were generated by partially covering a UV-B 313 tube (Q-Panel Co., U.S.A.) with dark coloured paper.

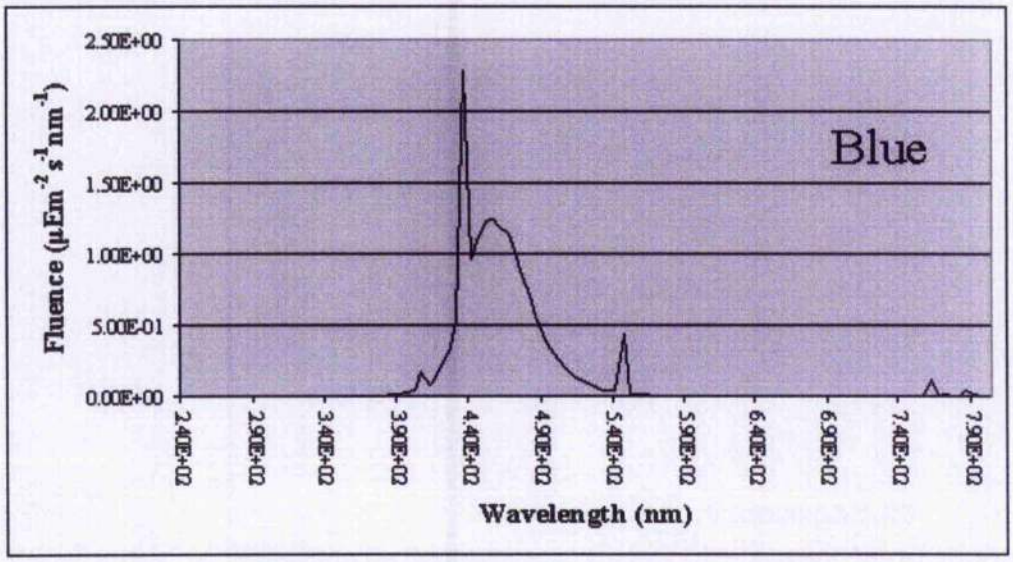
2.4.1.2 Fluence Rate Measurement

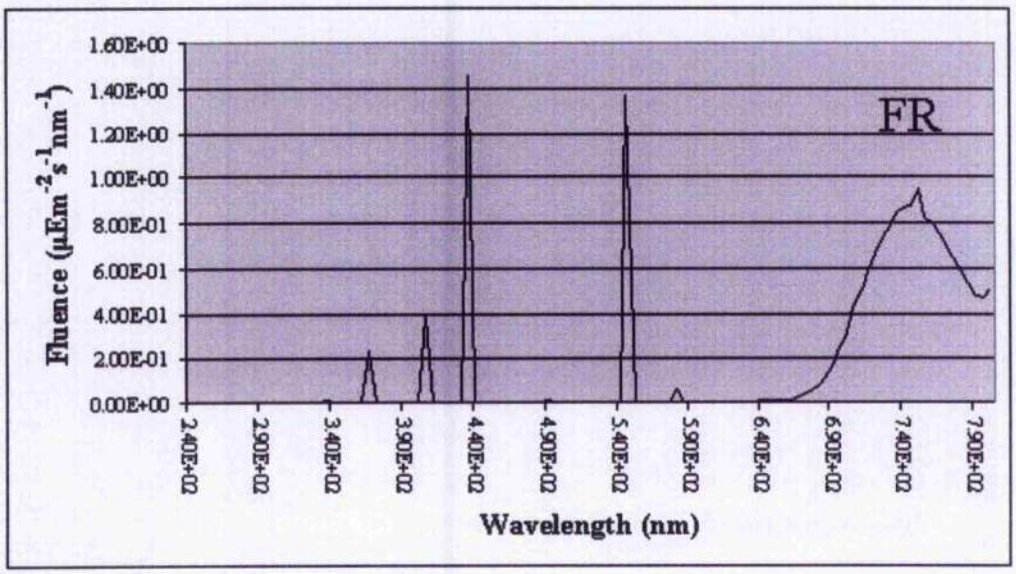
The photon fluence rates of white light were generally measured using a Skye RS232 meter fitted with a quantum sensor which measures photosynthetically active radiation (ie. 400-700 nm light). A hand held RS232 meter, Skye Instruments (Powys, Wales), was used to measure UV-A (315-380 nm) with an SKU 420 sensor, and UV-B (280-315 nm) with an SKU 430 sensor. In addition, a Macam Spectroradiometer Model SR9910 (Macam Photometrics Ltd., Livingston, Scotland) capable of measuring all wavelengths between 240 and 800 nm was used.

Figure 2.1
Spectra of light qualities at fluence rates used in some of the key experiments described in the text. These include 3 $\mu Em^{-2}s^{-1}$ UV-B (UV-B); 100 $\mu Em^{-2}s^{-1}$ white light (HW); 100 $\mu Em^{-2}s^{-1}$ UV-A (UV-A); 80 $\mu Em^{-2}s^{-1}$ blue (Blue); 70 $\mu Em^{-2}s^{-1}$ far-red (FR). The spectral photon distribution of each of these light qualities was measured using a Macam Spectroradiometer SR9910 (Macam Photometrics Ltd., Livingston, Scotland). Note that the y axes differ in scale.




UV-A
Fluence (µEm-2s-1nm-1)
6.00E+00
5.00E+00
4.00E+00
3.00E+00
2.00E+00
1.00E+00
0.00E+00
2.40E+02
2.90E+02
3.40E+02
3.90E+02
4.40E+02
4.90E+02
5.40E+02
5.90E+02
6.40E+02
6.90E+02
7.40E+02
7.90E+02
Wavelength (nm)


Blue
Fluence (µEm-2s-1nm-1)
2.50E+00
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
2.40E+02
2.90E+02
3.40E+02
3.90E+02
4.40E+02
4.90E+02
5.40E+02
5.90E+02
6.40E+02
6.90E+02
7.40E+02
7.90E+02
Wavelength (nm)


FR
Fluence (µEm-2s-1nm-1)
1.60E+00
1.40E+00
1.20E+00
1.00E+00
8.00E-01
6.00E-01
4.00E-01
2.00E-01
0.00E+00
2.40E+02
2.90E+02
3.40E+02
3.90E+02
4.40E+02
4.90E+02
5.40E+02
5.90E+02
6.40E+02
6.90E+02
7.40E+02
7.90E+02
Wavelength (nm)

2.4.2 Cold Treatment of Compost Grown Plants

Plants were grown for three weeks under low white light as described in Section 2.3.2 before a cold treatment of 24 hours at 7-10 °C in low white light was applied. Mature leaf tissue was then harvested, also as described in Section 2.3.2.

2.4.3 Assessing Supplementary UV-B Induced Growth Inhibition and Leaf Damage

The sensitivity of *Arabidopsis* plants to growth inhibition and leaf damage resulting from the adverse effects of supplementary UV-B illumination was assessed according to the method developed in Chapter Three (Section 3.6). Seedlings were grown on compost under 120 $\mu Em^{-2}s^{-1}$ white light for 12 days before treatments of the durations described with 5 $\mu Em^{-2}s^{-1}$ UV-B (supplemented with 40 $\mu Em^{-2}s^{-1}$ white light). Digital photographs were taken to illustrate the damage sustained by plants 5 days after recovery in 120 $\mu Em^{-2}s^{-1}$ white light.

2.4.4 Inhibition of Hypocotyl Elongation Experiments

Inhibition of *Arabidopsis* hypocotyl elongation in response to UV-B was measured in etiolated seedlings. Seeds were sterilized as described in Section 2.3.3 and germination was induced with a 3 hour pulse of 150 $\mu Em^{-2}s^{-1}$ white light before seedlings were grown on sucrose-containing media, in darkness for 2 days. Thereafter plates were kept in darkness or exposed to a further treatment with 0.25 $\mu Em^{-2}s^{-1}$ UV-B or equivalent 'UV-B Minus' (ie. lacking only the UV-B wavelengths) for 4 days. Fluence rates were measured through Petri dish lids as UV-B transmission through plastic can be significantly reduced. Seedling hypocotyl lengths were measured and the data illustrated (see Figure 3.3).

2.4.5 Histochemical Assay for β-Glucuronidase Production

Production of the reporter enzyme β-Glucuronidase was assayed histochemically. The cyclohexylammonium salt of X-Gluc (ie. 5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronide; catalogue No. MB1021) supplied by Melford Labs. (Chelsworth, Suffolk, U.K.) was dissolved in N,N-Dimethylformamide (Sigma, Poole, Dorset) to make a 25 mg ml^{-1} stock solution and stored in a light-tight container at -20 °C. Plant tissue was completely immersed in 1 mg ml^{-1} (final concentration) of X-Gluc, 50 mM $NaPO_4$ pH7, 0.1% (v/v) Triton X-100. Open vials were placed in a speed-vac apparatus (Kartell®, Italy) and a vacuum applied (3 x 2 minutes) to enhance infiltration of X-Gluc into plant tissue. Vials were then sealed, covered with foil and incubated overnight at 37 °C until the blue product of the enzyme reaction became visible. Chlorophyll was removed by immersing tissue in increasing concentrations of an ethanol solution.

2.4.6 Digital Photography

Digital photography was carried out using a Nikon Coolpix 995 3.34 Megapixel digital camera (Nikon, Tokyo, Japan) and Nikon View 4 software.

2.5 Isolation of Total RNA from Plant Material

The Flowgen Purescript RNA isolation kit (Flowgen, Staffordshire, U.K.) was used to extract total *Arabidopsis* RNA according to the manufacturer's instructions. Not more than 0.4 g of frozen tissue was ground to a fine powder using liquid nitrogen and a mortar and pestle, before being added to 300 µl of Flowgen Cell Lysis Solution (containing citric acid, diaminoethanetetra-acetic acid (EDTA) and SDS) in a 1.5 ml Eppendorf®. The tube was inverted and then vortexed to mix the contents before the addition of 100 µl of Flowgen Protein-DNA Precipitation Solution (containing citric acid and NaCl). The tube was gently inverted 10 times and placed in an ice bath for 5 minutes before centrifugation (15,000 *g* for 3 minutes) to pellet the precipitated proteins and DNA. The supernatant was next extracted

against 500 µl of chloroform with a 5 minute spin (15,000 *g*) and added to a fresh Eppendorf® containing 300 µl of isopropanol. After thoroughly mixing (50 inversions) and further centrifugation (15,000 *g* for 3 minutes) the RNA pellet was washed with 70 % ethanol and dried for 15 minutes. Finally, 30 µl of DEPC treated water was used to rehydrate the pellet which was stored at –80 °C. RNA was mixed thoroughly before use.

2.6 DNA Methods

2.6.1 Plant Genomic DNA Extraction from *Arabidopsis*

Genomic DNA was isolated from *Arabidopsis* leaf tissue using the DNeasy® Plant Mini Kit (Qiagen, Crawley, West Sussex, U.K.) all according to the manufacturer's instructions. Plant tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle, then transferred to a 1.5 ml Eppendorf® containing 400 µl of Buffer AP1 and 4 µl RNase A stock solution (100 mg ml^{-1}) and vortexed vigorously. The tube was incubated for 10 minutes at 65 °C and inverted 2-3 times during incubation to lyse the plant cells. Next, 130 µl of Buffer AP2 (containing acetic acid) was added to the lysate, mixed, and incubated for 5 minutes on ice. The lysate was applied to a QIAshredder spin column (lilac) sitting in a 2 ml collection tube and centrifuged for 2 minutes at 15,000 *g*. The flow through was then transferred to a fresh 1.5 ml Eppendorf® without disturbing the cell-debris pellet. Next, 1.5 volumes of Buffer AP3/E (containing guanidine hydrochloride) was added to the cleared lysate and mixed in by pipetting. Six hundred and fifty microlitres of mixture, including any precipitate which may have formed, was then added to the DNeasy® mini spin column sitting in a 2 ml collection tube (supplied). The column was centrifuged for 1 minute (15,000 *g*) and the flow-through discarded before the remainder of the sample was also passed through the column in the same way. The DNeasy® column was placed in a new 2 ml collection tube (supplied) and 500 µl of Buffer AW passed through the column (centrifuged for 1 minute at 15,000 *g*). The flow-through was discarded and the collection tube reused as 500 µl of Buffer AW was again

passed through the column, this time centrifuging for 2 minutes (15,000 *g*) to dry the membrane. Finally, the DNeasy column was transferred to a fresh 1.5 ml Eppendorf® and 100 µl of preheated (65 °C) Buffer AE pipetted directly onto the DNeasy® membrane. After incubating at room temperature for 5 minutes the genomic DNA was eluted into the tube by centrifugation (1 minute at 15,000 *g*). The elution step was repeated once as described, to increase the quantity of DNA recovered.

2.6.2 Digestion of DNA with Restriction Endonucleases

DNA restriction, synthesis and modification enzymes were used in accordance with the manufacturer's instructions. DNA was prepared in a 1x solution of the appropriate buffer (as recommended by the supplier) and approximately 5-20 units of restriction enzyme added to a concentration not exceeding 10 % (v/v). Reactions were incubated at the appropriate temperature overnight or for the required duration.

2.6.3 Oligonucleotide Primers

The oligonucleotide primers used in this work were synthesized by TAG Newcastle Ltd and distributed by VH Bio Ltd. (both Gateshead, U.K.). Primers were designed visually according to recommendations such as those published online by Dr. Michael Blaber (http://wine1.sb.fsu.edu/bch5425/lect23/lect23.htm). Several sets of primers were tested as standard for each application or polymorphism and the best pair used in subsequent work. Polymerase chain reactions were carried out in DYAD DNA Engine PTC-220 and DNA Engine PTC-200 Peltier Thermal Cycler (Genetic Research Instrumentation, Braintree, Essex, U.K.) machines. Primers used in this work are shown in Table 2.2.

Name	Sequence of Primer Pair	Amplified Fragment Size	Description
CHS1	5′-ATCTTTGAGATGGTGTCTGC-3′ 5′-CGTCTAGTATGAAGAGAACG-3′	337 bp	Used in quantitative reverse transcriptase polymerase chain reactions to assess *CHS* steady-state transcript levels.
CHS2	5′-CATGTCGTCTTCTGCACTAC-3′ 5′-CACCATCCTTAGCTGACTTC-3′	700 bp	Tested for suitability in amplification of *CHS* from cDNA.
CHSfr	5′-TAAAGCTAGGACTAAAGGAAG-3′ 5′-CGTCTAGTATGAAGAGAACG-3′	99 bp	Tested for suitability in amplification of *CHS* from cDNA. Designed by Dr. Helena Wade (University of Glasgow). One of these primers was also used in the CHS1 primer pair (above).
ACTIN 2as	5′-CTTACAATTTCCCGCTCTGC-3′ 5′-GTTGGGATGAACCAGAAGGA-3′	500 bp	Used in quantitative reverse transcriptase polymerase chain reactions as a control to ensure equivalent amounts of total mRNA are represented in each gel lane (Fontaine et al., 2002).
HY5	5′-GCTGCAAGCTCTTTACCATC-3′ 5′-AGCATCTGGTTCTCGTTCTG-3′	404 bp	Used in quantitative reverse transcriptase polymerase chain reactions to assess *HY5* steady-state transcript levels.
UVR for1 / rev1	5′-TCGAGAGATTACGGATAGTG-3′ 5′-CCAGTAGAGGTAACTCTTTC-3′	796 bp	Used to determine the sequences of the novel *uvr8* mutant alleles described in Section 4.4.1.
UVR for4 / rev3	5′-AGTGCCTCAGAAGATTCAAG-3′ 5′-CACAGTTTACAACGCCCATG-3′	1044 bp	Used to determine the sequences of the novel *uvr8* mutant alleles described in Section 4.4.1.
nga1139	5′-TAGCCGGATGAGTTGGTACC-3′ 5′-TTTTTCCTTGTGTTGCATTCC-3′	114 bp	Simple Sequence Length Polymorphism (SSLP). Used to map *30E5* gene.
Cer 460 644	5′-AGCAGATTAGTAGATCGTGG-3′ 5′-AATAGTTGCATAGCCTCTGG-3′	267 bp	Simple Sequence Length Polymorphism (SSLP). Used to map *30E5* gene.
Cer 460 650	5′-CCACAAACTTAGAAATTAGG-3′ 5′-TCTCAACTATTACAACAACC-3′	398 bp	Simple Sequence Length Polymorphism (SSLP). Used to map *30E5* gene.
Cer 460 656	5′-TTGCGTAATTTGGTAGTTGC-3′ 5′-GCTAACATGGAAATTTGTCG-3′	168 bp	Simple Sequence Length Polymorphism (SSLP). Used to map *30E5* gene.
Cer 460 658	5′-AACGCTCCCTTCAATATTCC-3′ 5′-GTTCTTCCCCATTGATATCG-3′	208 bp	Simple Sequence Length Polymorphism (SSLP). Used to map *30E5* gene.
F11I11-22	5′-TAAGTGACAGAGTGCATGTG-3′ 5′-AAAATGGCCATGTAGTGGAC-3′	981 bp	Cleaved Amplified Polymorphic Sequence (CAPS) highlighted by the restriction enzyme *Bcc*I. Used to map *30E5* gene.
Cer 424 643	5′-CATCTCTAAGTAAACGGTTG-3′ 5′-AGAATTCTGGTTCTGACTAG-3′	1064 bp	Single Nucleotide Polymorphism (SNP) highlighted by the restriction enzyme *Tsp*509 I. Used to map *30E5* gene.
Cer 424 650	5′-AGTATCACTGAGTTGATAGG-3′ 5′-CGTTAGCAAACTATACGACC-3′	560 bp	Single Nucleotide Polymorphism (SNP) highlighted by the restriction enzyme *Tsp*509 I. Used to map *30E5* gene.
DHS1	5′-CAAGTGACCTGAAGAGTATCG-3′ 5′-AGAGAGAATGAGAAATGGAGG-3′	1668 bp	Cleaved Amplified Polymorphic Sequence (CAPS) highlighted by the restriction enzyme *Dde*I (Konieczny and Ausubel, 1993). Used to map *30E5* gene.

Table 2.2
Oligonucleotide primers used in this study.

2.6.4 DNA Sequencing and Sequence Comparisons

DNA sequencing was carried out by staff of the Sir Henry Wellcome Functional Genomics Facility (University of Glasgow). Polymerase Chain Reaction was used to synthesize DNA and the same primers (diluted to 3.2 μM) were provided to S.H.W.F.G.F. for the sequencing reactions. Promega (Southampton, U.K.) *Taq* DNA polymerase (catalogue No. M1861) was used to generate genomic DNA fragments from the *30e5* mutant referred to in Section 6.6.2 and *Pfu* DNA polymerase (Promega, catalogue No. M7741) was used to amplify cDNA fragments from the *uvr8* alleles described in Section 4.4.1. DNA sequence comparisons were performed using the Basic Local Alignment Search Tool (BLAST) provided by The National Center for Biotechnology Information (NCBI) online (http://www.ncbi.nlm.nih.gov/blast/) or using the complete *Arabidopsis* Landsberg ecotype genome sequence resource (Jander et al., 2002) previously administered by Cereon Genomics (now Monsanto Company, Missouri, U.S.A).

2.7 Agarose Gel Electrophoresis

The required amount of electrophoresis grade agarose (0.8-4 % (w/v)) was added to the necessary volume of 0.5 x TBE (44 mM Tris-borate, 1 mM disodium EDTA, pH 8.0) for the gel being cast. The suspension was heated in a microwave until the agarose had completely dissolved. High percentage gels must be heated carefully to avoid accidents – ideally, the gel should be slowly mixed on a magnetic stirrer between short periods of heating. The solution was allowed to cool to around 60 °C, whereupon ethidium bromide was added to a final concentration of approximately 0.3 μg ml^{-1} and the gel solution poured into a casting tray. After the gel had set it was placed in the electrophoresis apparatus and submerged in 0.5 x TBE running buffer. DNA aliquots / reactions were mixed with 1/6 volume of loading buffer (50% (v/v) glycerol, 1 mM disodium EDTA pH 8, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 0.25% (w/v) orange G) and applied to the gel with a pipette. Electrophoresis was allowed to proceed at 20-120 mA until the DNA molecules had been

clearly separated. The gel was then visualized under UV light (transilluminator model TFM-20; Ultra-Violet Products, Cambridge, U.K.) and photographed using a Polaroid (Waltham, Massachusetts, U.S.A) GelCam or a Bio-Rad (Hemel Hempstead, Hertfordshire, U.K.) Gel Documentation System with Quantity One® (Version 4.3.0) Software. DNA Molecular Weight Marker X (catalogue No. 1498037, Roche Diagnostics, Mannheim, Germany) was used to calibrate DNA fragment sizes.

2.8 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

2.8.1 DNase Treatment

Following extraction of total RNA, a DNase treatment (DNA-free, Ambion, Huntingdon, UK) was used to eliminate contamination with genomic DNA. The RNA was thawed, mixed and stored on ice before 2.5 µl (approximately 5 µg) was added to a sterile Eppendorf®. Next, 1 x DNase I Buffer (Ambion), 2 units of DNase I and sterile water to a total volume of 35.5 µl were mixed together gently with the RNA and incubated at 37 °C for 30 minutes. DNase Inactivation Reagent (Ambion) was then resuspended and 5 µl of slurry added to the RNA mix using a sterile cut-off pipette tip. The tube contents were mixed and the inactivation reaction was allowed to proceed for 2 minutes at room temperature with the tube being flick-mixed once during incubation. Microcentrifugation (15,000 *g* for 1 minute) was then used to pellet the DNase Inactivation Reagent. To ensure the DNase treatment had worked, 2.5 µl of the DNased RNA was 'amplified' for 35 cycles, along with a positive (*Arabidopsis* genomic DNA) and negative (sterile water) control. The ACTIN2as primers and PCR conditions described below were used to ensure no PCR product was generated from the DNased RNA under these conditions.

2.8.2 cDNA Synthesis

Ten microlitres of each DNased RNA sample (generated as described in Section 2.8.1) was added to a fresh Eppendorf® with 0.24 μM oligo dT ($dTTP_{15}$) and incubated at 70 °C for 10 minutes. The mixture was then cooled briefly on ice and the contents spun down (pulse spin). Next, 1 x AMV Reverse Transcriptase Reaction Buffer (Promega, Southampton U.K.), 1 mM of each dNTP (Promega, catalogue No. U1240), 24 Units of RNase inhibitor (Promega), 1 mM dithiothreitol, 10 Units of AMV Reverse Transcriptase (Promega) and DEPC water to a total volume of 25 μl were added and the reaction allowed to proceed at 48 °C for 45 minutes. Finally, the enzyme was inactivated at 95 °C for 5 minutes and the resulting cDNA stored at – 20 °C.

2.8.3 PCR Reactions

Quantitative RT-PCR can only be performed if DNA is generated in reactions which are terminated whilst amplification is still in the exponential phase. Twenty-two cycles were found to be ideal for *CHS* quantification and 24 cycles for *HY5* measurement. The ACTIN2as primers were effective at either 22 or 24 cycles. For quantitative RT-PCR the conditions used were: (2 mins 30 secs at 94 °C, 1 min at 55 °C, 2 mins at 72 °C) for one cycle; then (45 secs at 94 °C, 1 min at 55 °C, 1 min at 72 °C) all for 22 (*CHS*) or 24 (*HY5*) cycles; finally, 5 mins at 72 °C for one cycle. The quantity of cDNA (eg. 1 μl) added to each tube was adjusted until the ACTIN2as loading control primers showed that equivalent amounts of total RNA were being compared in each gel lane. Reaction ingredients were made up as a single master mix containing 1 x PCR Buffer, 1.5 mM $MgCl_2$ (Promega), 0.2 mM dNTPs, 0.5 μM of each of the four primers (ACTIN2as and either the CHS or HY5 primers), 0.625 Units of *Taq* DNA Polymerase (catalogue No. M1861, Promega) and sterile water to a final reaction volume of 25 μl. Reactions were carried out in the machines described in Section 2.6.3 and PCR products were separated and quantified on 2 % agarose gels as described in Section 2.7.

2.9 Screening for Mutants with Altered Responses to UV-B

2.9.1 Mutagenesis using Ethyl Methane Sulfonate (EMS)

Mutagenesis with Ethyl Methane Sulfonate (EMS) was carried out according to the procedure described by Leyser and Furner in The *Arabidopsis* Compleat Guide (ftp://ftp.arabidopsis.org/home/tair/Protocols/compleat_guide/comguidePDFs/6_EMS_mutagensis.pdf). The concentration of mutagen used was varied (for the *CHS-Luc* screen only) to try to ensure that mutagenesis was effective. Mutageneses were carried out in a fume cupboard and using a medium Atmos bag (catalogue No. Z112828; Sigma, Poole, Dorset). Approximately 15,000 (0.3 g) *Arabidopsis* seeds were pre-imbibed overnight in a solution of 0.1 % (w/v) potassium chloride. All apparatus was then transferred to the Atmos bag inside a fume cupboard and the seeds were soaked for 3 hours in 0.1 M sodium phosphate pH 5.0 (made by mixing Na_2HPO_4 and NaH_2PO_4 in the appropriate quantities), 5% (v/v) dimethyl sulfoxide, 0.6 % (v/v) EMS. The seeds were then washed twice in 100 mM sodium thiosulphate for 15 minutes and waste solutions were discarded into a beaker of solid sodium thiosulphate to inactivate the EMS. Next, the seeds were twice washed in distilled water for 15 minutes before the Atmos bag was opened and all waste solutions and plasticware placed in a sink under running water for 30 minutes. The mutagenized seeds were dried on filter paper overnight, before being sown on compost as described in Section 2.3.2. The progeny of these mutagenized M_1 plants, the M_2 seed, were collected in batches (approximately 40 M_1 plants per batch) and subsequently screened for mutations.

2.9.2 The *CHS-Luc* Screen

2.9.2.1 Bioluminescence Imaging and Photon Counting

Bioluminescence imaging and photon counting of *Arabidopsis* plants expressing transgenic luciferase was carried out according to the methods of Miller *et al.* and Michelet and Chua (Millar et al., 1992, Michelet and Chua, 1996), with adaptations introduced by G. R. Littlejohn (Division of Biochemistry and Molecular Biology, University of Glasgow). The

light-labile substrate for the luciferase reaction, beetle luciferin (catalogue No. E160C; Promega, Southampton, U.K.) was made up as a 150 mM stock solution in sterile water, dispensed into 50 µl aliquots and stored inside foil covered Eppendorfs® at –80 °C. A 5 mM luciferin working solution was made up in 0.01 % (v/v) Triton x-100 for pre-treatment spraying of plants. Pre-spraying with a high concentration of luciferin rids the plant of background luciferase, expressed during growth, since the luciferase is inactivated whilst generating bioluminescence. A Ding Hwa Co. Ltd. (Taipei, Taiwan) mini air compressor (Model AC-100) fitted with a standard artist's airbrush was used to apply the luciferin substrate solution to *Arabidopsis* plants grown (as described in Section 2.3.2) on compost. Two pre-sprays using 5 mM luciferin (in 0.01 % (v/v) Triton x-100) were performed to remove background luciferase 18 hours and 12 hours, respectively, before the light treatment was applied. Imaging sprays to assess the level of *CHS* promoter activity were performed using 1 mM luciferin (in 0.01 % (v/v) Triton x-100) and 20 minutes were allowed to elapse between spraying and imaging to permit the level of bioluminescence to plateau. Imaging sprays were performed both before and after light treatments where necessary.

Luciferase-expressing *Arabidopsis* plants were imaged using a Photek (East Sussex, U.K.) ICCD 225 photon counting camera (model 5874-1/2143-1) and IFS32 software in accordance with the manufacturer's instructions. The plant material was brought into focus inside an imaging chamber using the bright-field camera setting and the focus dial on the camera lens. The photon counting camera was then activated with the filter set at 100 % and a timed integration initiated for 6-12 minutes. Images (illustrated in Figures 4.2, 4.3 and 6.4) were saved and luciferase luminescence estimated visually or quantified electronically, depending on the particular procedure.

2.9.2.2 Preliminary Timecourse

Transgenic *Arabidopsis* plants containing the *CHS-Luc* reporter gene (both the *CHS-Luc 1.7* and the *CHS-Luc 3.4* lines) were grown on compost as described in Section 2.3.2 under 25 $\mu Em^{-2}s^{-1}$ white light for 15 days. Thereafter, 3 $\mu Em^{-2}s^{-1}$ UV-B was applied for 0-12 hours each to a separate tray of plants (ie. 0 hrs, 1 hr, 2 hrs UV-B etc.). Photon counting imaging was performed as described in Section 2.9.2.1 with images being recorded before and after the light treatment was applied to each tray. These images were quantified using the Photek software and photons counted within an equivalent area in each image. The level of *CHS* expression induced by each treatment per seedling was calculated by subtracting the luminescence recorded in each pre-treatment image from the luminescence in the same area of the corresponding post-treatment image and this figure was divided by the total number of seedlings present in that particular tray (all as per the method of G. R. Littlejohn, University of Glasgow). The results are shown in Figure 4.1.

2.9.2.3 Screening for Mutants Altered in UV-B Induced *CHS* Expression Levels

Arabidopsis seeds from the *CHS-Luc 3.4* line were mutagenized as described in Section 2.9.1. M_2 seedlings were grown on compost under 20-35 $\mu Em^{-2}s^{-1}$ white light and screened after 14 days for alterations in the level of UV-B (3-4 hours treatment with 3 $\mu Em^{-2}s^{-1}$) induced *CHS-Luc* expression as described in Section 2.9.2.1. Seedlings with altered levels of *CHS* induced luciferase production were identified and carefully transplanted to a fresh tray of compost for collection of M_3 seed from each plant separately. Several M_3 seeds from each putatively altered M_2 plant were sown together on compost and the screening procedure repeated to look for consistent alterations in UV-B induced *CHS-Luc* expression. The progeny of single M_2 plants which appeared to be altered in reporter gene expression levels were then grown for three weeks under 18-35 $\mu Em^{-2}s^{-1}$ white light before RNA extraction and qRT-PCR were performed as described previously (Sections 2.5 and 2.8) to assess the endogenous *CHS* expression level found in each putative mutant after (a) no further treatment, (b) 6 hours of 100 $\mu Em^{-2}s^{-1}$ UV-A or (c) 4 hours of 3 $\mu Em^{-2}s^{-1}$ UV-B treatment.

2.9.3 The Phototropism Screen

As detailed in Chapter Five, the preliminary work which laid the foundations for the phototropism screen experimented with variations in UV-B fluence rate and treatment duration, seedling age and growth media content. Mutageneses of *phot1-5phot2-1* seeds were carried out as described in Section 2.9.1. Sterilization of the *phot1-5phot2-1* M_2 seeds for the phototropism screen was performed according to the method described in Section 2.3.3.2 with minor modifications. After seeds were resuspended in 0.1% (w/v) sterile agarose, up to 200 µl of the resuspension was carefully pipetted, using a cut-off pipette tip, along the surface of a sterile, wet, filter paper strip according to a modification of the basic method which was added by Dr. J. M. Christie (University of Glasgow). The filter paper strips had been arranged horizontally across each Petri dish which contained growth media including 2 % (w/v) sucrose (see Section 2.3.4). Square Petri dishes (100 mm x 100 mm manufactured by Bibby Sterilin Ltd., Staffordshire, U.K.) were used for the phototropism screen so that these could stand upright in the light treatment chambers and Micropore® tape used to seal each plate was applied to the top and bottom edges only in order to allow the directional UV-B to penetrate unobstructed. Germination was induced using 1-2 hrs of 50 $\mu Em^{-2}s^{-1}$ red or white light and the seedlings grown upright (with the plates standing on their edge) for a further 60 hours in complete darkness before treatment with 0.5 $\mu Em^{-2}s^{-1}$ directional UV-B (measured through a Petri dish lid) for 48 hrs. Healthy, non-phototropic M_2 seedlings were washed and carefully transferred to compost for collection of M_3 seed from each individual M_2 putative mutant. M_3 seed from single M_2 *phot1phot2* putative mutants was tested in quantity and those which remained non-UV-B-phototropic were retained. Finally, two plates of each potential mutant were carefully reassessed as before (but on separate occasions) compared to the *phot1phot2* parent line. Digital photographs of these comparisons were recorded.

2.10 Map-Based Cloning

Map-based cloning was approached in such a way as to take full advantage of recent developments in the field (Jander et al., 2002). An F_2 mapping population consisting of 1000 DNA samples, each one corresponding to a single plant was created by selecting *30e5* mutant phenotype individuals from a segregating population of *30e5* (L. *er* background) x Col3 F_2 plants. Seedlings were grown on compost and harvested (as described in Section 2.3.2) for DNA extractions (described in Section 2.6.1). Plants were grown under approximately 150 $\mu Em^{-2}s^{-1}$ to enhance the chlorotic *30e5* phenotype, thereby allowing the mutants to be selected.

A fine-scale mapping population of 66 DNA samples, each one corresponding to a single plant containing a recombination breakpoint between *30E5* and the neighbouring *FAH1* gene was created by crossing the *30e5* (L. *er*) and *fah1* (Columbia) mutants together. Only *30e5* phenotype plants were retained from the F_2 population derived from this cross and seed was collected from each of the plants retained, individually. The resulting F_3 seed from each F_2 plant was sown separately and those F_3s showing segregation for the *fah1* phenotype – a red fluorescence under UV light (transilluminator model TFM-20; Ultra-Violet Products, Cambridge, U.K.) identified. A single *30e5fah1* double mutant plant from each F_2 which gave rise to progeny with both recessive mutant phenotypes was retained for DNA extraction, performed as described in Section 2.6.1.

PCR-based markers highlighting polymorphisms between the Landsberg and Columbia ecotypes were identified in the literature (Konieczny and Ausubel, 1993, Bell and Ecker, 1994), online (http://www.arabidopsis.org/servlets/Search?action=new_search&type=marker) and from the Cereon *Arabidopsis* Polymorphism Collection (http://www.arabidopsis.org/cereon/). Primers were synthesized as described in Section 2.6.3 and tested for efficacy. High percentage agarose gels, 4 % (w/v), were poured as described in Section 2.7 to separate small DNA molecules, similar in size. The polymorphic markers described in Table 2.2 were used to identify the closest possible flanking markers on either

side of the *30E5* gene. The location of the *30E5* gene together with the relative positions of the closest markers is shown in Figure 6.8.

2.11 Affymetrix Microarray

Mature *Arabidopsis* were grown on compost for 3 weeks under low white light as described in Section 2.3.2 and then treated, or not treated, with 4 hours of 3 $\mu Em^{-2}s^{-1}$ UV-B before harvesting. Total RNA was extracted as described in Section 2.5. The remaining procedures in this section were carried out by staff of the Sir Henry Wellcome Functional Genomics Facility at the University of Glasgow. Five micrograms of total RNA was reverse transcribed using the SuperScript II system (Invitrogen, Paisley, UK). The oligonucleotide used for priming was 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(T)_{24}$-3', as recommended by Affymetrix (Santa Clara, U.S.A; see 'Eukaryotic Sample and Array Processing' http://www.affymetrix.com/support/downloads/manuals/expression_s2_manual.pdf). Double-stranded cDNA was purified by phenol / chloroform / isoamylalcohol extraction and the aqueous phase removed by centrifugation through Phase-lock Gel (Eppendorf, Histon, Cambridge, U.K.), followed by pellet paint precipitation and resuspension of the double stranded cDNA in nuclease free water. *In vitro* transcription was performed on 5 µl of the resulting cDNA using the Affymetrix GeneChip labelling kit (Affymetrix, Santa Clara, U.S.A). The biotinylated cRNA was next purified using RNeasy clean-up mini-columns (Qiagen, Crawley, UK). The first and second eluates (each 30 µl) were collected separately and 19.2 µg of biotinylated cRNA from the first eluate was fragmented to prepare probe by heating in 1x fragmentation buffer (40 mM Tris-acetate, pH 8.1 / 100 mM KOAc / 30 mM MgOAc) as recommended by Affymetrix (note that Dimethyl Sulfoxide was not used for probe preparation). Probes were hybridized to Affymetrix *Arabidopsis* ATH1 GeneChips in a hybridization oven using the standard Affymetrix procedure (45°C at less than 1 *g* for 16 hrs).

Washing and staining were performed in a Fluidics Station 400 (Affymetrics) using the protocol EukGE-WS2v4; the chips were stained with Streptavidin Phycoerythrin (SAPE) followed by biotinylated antibody followed by SAPE again. The GeneChips were subsequently scanned in a Gene Array Scanner 2500 and the data analyzed using FunAlyse Version 1.2 software. Low level normalization was done using the Robust Multichip Average (RMA) method (Irizarry et al., 2003) implemented in the module 'Affy' of the 'Bioconductor' microarray analysis software, and differentially expressed genes identified using the RankProducts (RP3) method (Breitling et al., 2004). Results were obtained in triplicate to ensure statistical significance, and for further analysis the differentially expressed gene lists were cut at a false discovery rate of 5% (Storey, 2003).

CHAPTER 3

THE DEVELOPMENT OF SCREENS FOR THE ISOLATION OF *Arabidopsis* MUTANTS ALTERED IN THEIR RESPONSE TO ULTRAVIOLET-B LIGHT

3.1 Introduction

The genetic approach is one of the most powerful tools in biology and can provide key insights into the operation of many biological processes. However, performing an exhaustive screen for mutants is nearly always both lengthy and labour intensive. It is therefore critical, if resources are not to be wasted, to ensure that the proposed screen is practical and has the power to generate mutants of the kind sought. Indeed, so important is it to ensure that a solid foundation is laid for isolating mutants, that months rather than weeks should normally be spent exploring the best possible experimental conditions and treatments for a mutant screen.

3.2 General Considerations For Undertaking Screens to Isolate *Arabidopsis* Mutants Altered in their Response to UV-B Light

3.2.1 Care Should be Taken to Ensure that Each Response Under Study Depends Upon UV-B Wavelengths (ie. 280-320 nm) of Light

Since the aim of the present work is to isolate and characterize mutants which have lost (or are altered in) their response to UV-B light treatment, it must first be demonstrated that the responses being investigated are indeed responses specifically to UV-B.

It is important to remember that the UV-B fluorescent tubes used in the present work emit significant quantities of other wavelengths of light (as shown in Figure 3.1). Therefore, to confirm that the responses under study were dependent specifically on UV-B (ie. 280-320 nm) we tried to determine whether or not these responses were lost when a 'Clear 130' filter

(Lee Filters, Andover) which removes UV-B wavelengths was applied to the UV-B fluorescent tubes. Typical spectra from our fluorescent tubes both without (designated 'UV-B Plus' treatment) and with (designated 'UV-B Minus' treatment) the filter applied are shown in Figure 3.1. By comparison, Figure 3.2 shows corresponding 'UV-B Plus' and 'UV-B Minus' spectra for UV-B supplemented with white light used in our investigations into increases in the extent of leaf damage and plant growth inhibition produced as a result of ultraviolet-B treatment. For the purposes of screening, we should remember that phenotypes seen in wild-type plants after light treatments lacking UV-B wavelengths (ie. 'UV-B Minus' treatments) may resemble phenotypes seen in mutant plants which have lost the ability of the wild-type to respond to UV-B.

It should also be noted that many responses of *Arabidopsis* to UV-B (eg. inhibition of hypocotyl elongation, *CHS* gene expression and phototropism) can additionally be triggered by UV-A / blue wavelengths of light. The filter used in the present work to eliminate UV-B, transmits UV-A / blue wavelengths (with blue being transmitted better than UV-A). Care was taken to try to ensure that 'UV-B Minus' control treatments delivered at least an equivalent dose of UV-A / blue, ensuring that any 'UV-B Plus' treatment responses observed could not be attributed to higher UV-A or blue light fluence rates.

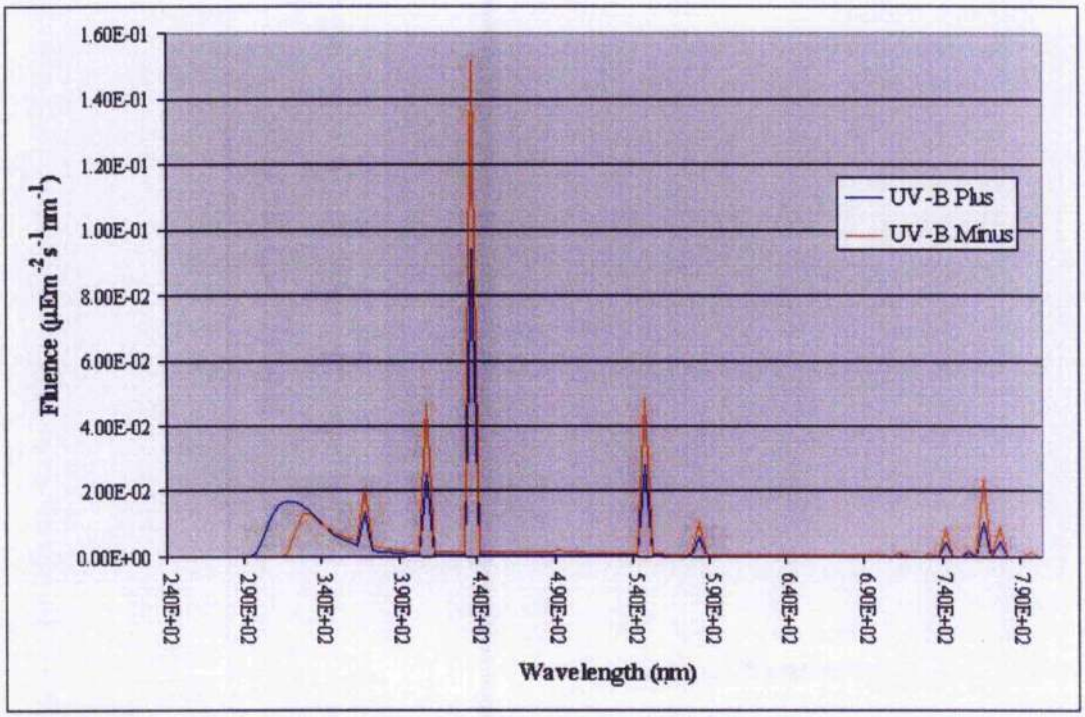


Figure 3.1
Spectra from UV-B 313 Fluorescent Tube Producing 0.5 $\mu Em^{-2}s^{-1}$ UV-B Without (Minus) and With (Plus) UV-B Wavelengths.

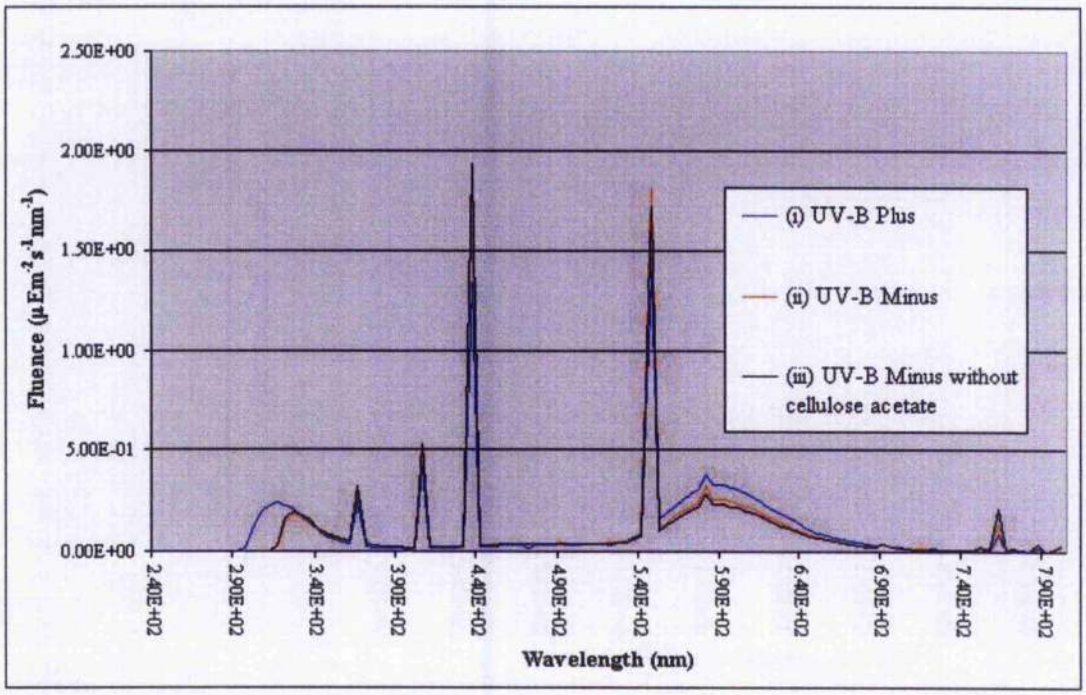


Figure 3.2
Spectra Generated During a UV-B Sensitivity Pilot Experiment (i) 5 $\mu Em^{-2}s^{-1}$ Supplementary UV-B, (ii) Minus UV-B and (iii) Minus UV-B without Cellulose Acetate. The spectral photon distribution in each of the light conditions shown above (Figures 3.2 and 3.3) was measured using a spectroradiometer (Macam SR9910) at the UV-B light intensities indicated. The third spectrum (iii) in Figure 3.2 was made to control for the loss of some of the cellulose acetate filter during this experiment.

3.2.2 Each Screen Should be Conducted Using a UV-B Fluence Rate Which Generates the Desired Response

In addition to showing that each particular response can be elicited specifically by UV-B, work should be done to determine the UV-B fluence rates and treatment durations which provide the clearest and most consistent responses possible.

Different types of UV-B response eg. photomorphogenesis, biomolecular damage, pigment production, DNA repair, etc. are most apparent under different fluence rates of UV-B so the optimal fluence rate should be determined for each response under study separately. As noted previously, finding mutants altered in UV-B perception or signalling may require screening under low doses of non-supplementary UV-B which are sufficient to induce photomorphogenesis but not sufficient to generate DNA damage (Ballare et al., 1991, Kim et al., 1998, Suesslin and Frohnmeyer, 2003).

3.2.3 The Screen Should be Capable of Producing Unambiguous Results Which Reflect Genetic Mutations

Variation is a pervasive theme in biology. Not long after the rediscovery, in 1900, of Gregor Mendel's principles of inheritance it was realised that variation has a particulate basis. The particles of heredity we now call 'genes', a term introduced by the Danish botanist and geneticist Wilhelm Johanssen in 1909 (Price, 1996, Muir, 1994).

When designing screens for mutations in genes, it is essential that a clear difference between mutant and non-mutant phenotypes can be observed. Unless a screen is simple and produces clear results, it is unlikely to be successful. Consequently, it should be possible to select M_2 plants with unambiguous mutant phenotypes and to show that the same unambiguous mutant phenotypes are inherited by the M_3 progeny. If map-based cloning is being contemplated then a clear (preferably visible) mutant phenotype is extremely important as hundreds, perhaps thousands, of mutant plants must be reliably selected from amongst their non-mutant counterparts if the mutant gene is to be located.

3.2.4 Screens Should be Geared Towards Producing Novel Rather Than Existing Mutants

Screens should be designed in such a way as to enhance the likelihood of isolating specific categories of novel mutant. The best way to ensure that large numbers of existing mutants are not produced from a screen is probably to focus upon a UV-B response associated with few existing mutants, for example *CHS* expression in *Arabidopsis*. Another way to circumvent the problem of existing mutants is to incorporate these into the screen to begin with (ie. instead of simply using a wild-type background). One mutant previously used as the parent line in a screen is *tt5*, which lacks the flavonoid biosynthetic enzyme chalcone isomerase. Kliebenstein *et. al.* used L. *er tt5* as the basis of a screen for mutants which showed a further enhancement of susceptibility to growth impairment and leaf damage induced by supplementary UV-B. One of the resulting mutants was called *uvr8* (Kliebenstein et al., 2002). Similarly, our own UV-B phototropism screen uses the *phot1phot2* double mutant as its starting point, thereby ensuring that neither phototropin can contribute to any response observed nor correspond to any mutant isolated.

Adjusting screening conditions (eg. the fluence rate of a particular light treatment) is another way to tailor a screen in order to make the generation of some categories of mutant more likely than others. Regardless of these precautions however, it may be necessary to cross-pollinate putative mutants arising from a screen with mutants generated in the past, studying the progeny in order to confirm that the newly isolated mutants are not defective in genes allelic to those previously identified.

3.2.5 Putative Mutants Must be Capable of Being Rescued from the Screen Conditions

It is clearly important that M_2 individuals, isolated as putative mutants, survive to generate a quantity of M_3 seed which can be used to look again for the mutant phenotype in a significant number of plants. Should the screening conditions be deleterious to the health of the mutants, plant survival could be threatened and so it is critical that a sensible rescue procedure has been developed in order to prevent the loss of valuable mutants. A difficult compromise may have to be reached to generate enough stress to display the mutant phenotype without killing the plants outright. Additionally, it is important to consider that many plant screening procedures are carried out on agar plates and transferring seedlings from these plates to compost (for eventual seed production) requires skill and care.

3.3 A Series of Pilot Experiments Suggested that it May be Difficult to Develop a Practical Screen for *Arabidopsis* Mutants Altered in the Inhibition of Hypocotyl Elongation and / or the Opening of Cotyledons in Response to UV-B Light

Although the onset of the developmental program, called photomorphogenesis, which controls plant growth in sunlight is thought usually to be triggered by phytochromes and cryptochromes, certain aspects of this program, such as the inhibition of hypocotyl elongation and cotyledon expansion, can also be induced by low fluence rate (<1 $\mu Em^{-2}s^{-1}$) UV-B (Kim et al., 1998). Recent results in *Arabidopsis* have suggested that this low fluence rate, UV-B induced photomorphogenesis is mediated by something other than the known photoreceptors (Suesslin and Frohnmeyer, 2003, Frohnmeyer and Staiger, 2003, Boccalandro et al., 2001) and one of the first investigations I undertook, as part of the present work, involved a series of pilot experiments designed to determine whether it would be feasible to screen for *Arabidopsis* mutants which had lost the ability to trigger photomorphogenesis in response to UV-B light. Two types of UV-B fluorescent tube were tested for their suitability during the course of this work.

Pilot experiments generally involved 2-day-old, dark grown (on Growth Media containing sucrose or, alternatively, compost) *Arabidopsis* seedlings being treated for 3-5 days in the dark, in low fluence rate UV-B, or in an identical light regime lacking the UV-B wavelengths respectively. Plants were then examined for signs of photomorphogenesis (ie. inhibition of hypocotyl elongation and cotyledon expansion). Various wild-type ecotypes, mutants and mutant combinations were tested to see which, if any, showed a discernible increase in the extent of photomorphogenesis produced when UV-B wavelengths were added to treatments as shown in Figure 3.3. However, only the *cry1cry2* double mutant appeared to show a repeatable UV-B specific increase in photomorphogenesis (see Figure 3.3 below) and this increase was deemed to be too small (approximately 2 mm ie. 10-20% of the untreated hypocotyl length) to form the basis for a reliable screen.

There is an additional reason to believe that screening for mutants which lacked UV-B induced inhibition of hypocotyl elongation would have been unwise: Comparing hypocotyl lengths of dark grown seedlings with those of seedlings treated with light lacking only the UV-B wavelengths demonstrates that non-UV-B wavelengths play a significant role in the hypocotyl inhibition response. This may be a problem even in the *cry1cry2* double mutant background as there is variable shortening in *cry1cry2* in response to 'UV-B Minus' treatment (data not shown).

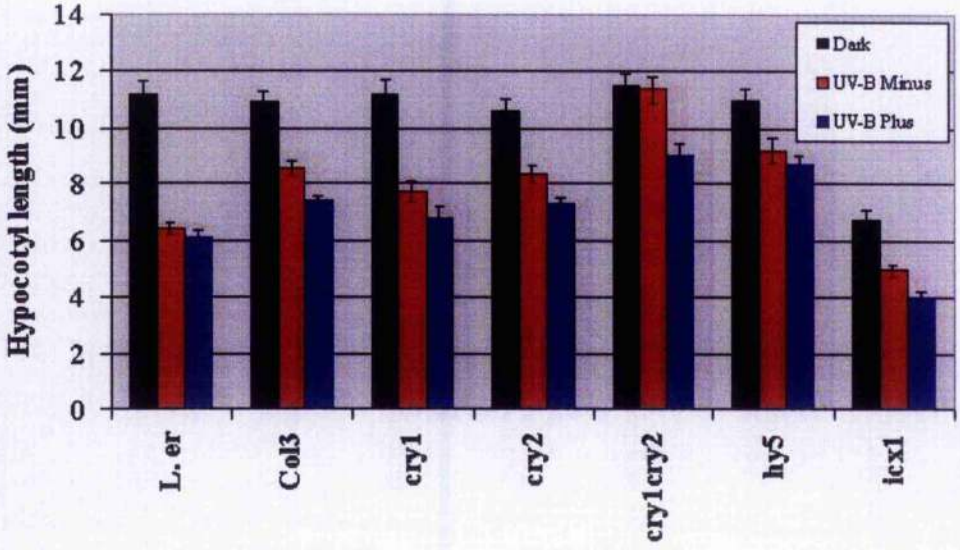


Figure 3.3
Inhibition of *Arabidopsis* Hypocotyl Elongation in Response to UV-B (0.25 $\mu Em^{-2}s^{-1}$) Wavelengths Specifically. Hypocotyl length of 2-day-old *Arabidopsis* seedlings, including various mutants, kept in darkness or exposed to a further 4 day treatment with 0.25 $\mu Em^{-2}s^{-1}$ UV-B or equivalent 'UV-B Minus'. Error bars give standard errors (n=20 for each condition).

3.4 The Use of a Transgenic Background Line Containing a *CHS* Promoter Linked to a Luciferase Reporter Gene Resulted in a Successful Screen for *Arabidopsis* Mutants Altered in the UV-B Induced Expression of Chalcone Synthase

The enzyme chalcone synthase (CHS) catalyzes the first committed step in flavonoid biosynthesis. Flavonoids are a large group of compounds with a wide functional diversity; many have important roles in shielding the plant from biotic and abiotic stress. Endogenous CHS enzyme accumulation is controlled primarily by transcription of the *CHS* gene and steady-state transcript levels are increased by blue, UV-A and UV-B light. The cryptochrome photoreceptors, cry1 and cry2, have been shown to mediate UV-A / blue light induction of *CHS* expression, but the photoreceptor(s) by which the UV-B signal is perceived remain unknown (Jenkins et al., 2001). Previous work has demonstrated the UV-B specificity of one particularly effective *CHS* induction pathway in *Arabidopsis* (Christie and Jenkins, 1996), and has also shown that *CHS* induction *via* this pathway is not attenuated in the *cry1cry2* double mutant (Wade et al., 2001). In order to try to identify perception / signalling components involved in mediating UV-B induced *CHS* expression, we used a genetic approach based on an *Arabidopsis* line which differed from the wild-type L. *er* ecotype only insofar as it contained an additional, single transgene consisting of a *CHS* promoter fused to a luciferase (*LUC*) reporter. Several mutants altered in UV-B induced *CHS* gene expression were isolated whilst taking advantage of the reporter gene to screen mutagenized M_2 plants; a detailed account of this screen and its results is provided in Chapter Four.

3.5 A Screen for Mutants Lacking a UV-B Induced Phototropic Response was Initiated in the *phot1phot2* Double Mutant Background

Phototropism is the process by which seedlings, plants or plant organs reorient themselves in response to lateral differences in light quality or quantity; phototropic hypocotyl curvature in response to UV-A / blue wavelengths is known to be mediated by the phototropin photoreceptors (phot1 and phot2). We have been able to show that UV-B induces positive phototropic hypocotyl curvature in the *Arabidopsis phot1phot2* double mutant, thereby demonstrating the existence of a UV-B sensitive phototropic response which is not dependent on either of the phototropins. In order to develop a screen for mutants of the associated UV-B stimulated phototropism signalling pathway using the *phot1phot2* double mutant background, various aspects of the treatment conditions (eg. UV-B fluence rate, treatment duration, seedling age and media content) were adjusted; a detailed account of the development and results of this screen is provided in Chapter Five.

3.6 Pilot Experiments Demonstrated that a Screen for Mutants with Enhanced Susceptibility to Supplementary UV-B Induced Growth Inhibition and Leaf Damage Could Provide Mutants Which May Help us Understand how *Arabidopsis* Responds to UV-B

3.6.1 Mutants Which Could Prove to be of Particular Interest to Our Work Might be Isolated on the Basis of their Enhanced Sensitivity to UV-B Induced Leaf Damage

As noted in Chapter One, a very striking response of mature *Arabidopsis thaliana* tissue to high fluence rate UV-B is that the plant sustains damage. Such UV-B induced damage usually manifests itself visibly in two ways, namely a reduction in overall plant growth rate and an increase in leaf necrosis. Now, an *Arabidopsis* mutant called *uvr8*, which turned out to have great significance for our work, had been isolated in a screen for mutants which were hypersensitive to UV-B induced leaf damage, before it was discovered that the *uvr8* mutant also shows a marked reduction in UV-B induced *CHS* gene expression (Kliebenstein et al., 2002). Because the significance of the *uvr8* mutant for our work was becoming increasingly apparent, and also because characterizing other mutants in which we had an interest overlapped with UV-B sensitivity work, we considered initiating a screen for *Arabidopsis* mutants which showed enhanced susceptibility to supplementary UV-B induced growth inhibition and leaf damage.

3.6.2 The Likelihood that Proceeding with a Screen for Mutants Sensitive to Supplementary UV-B Induced Leaf Damage Will Meet with Success Must be Weighed Against the Potential Pitfalls

Our experience of looking for small numbers of mutants amongst very large numbers of non-mutant plants suggests that initiating a simple screen for a visible response is attractive in prospect. Some of the biggest problems we have encountered when applying the genetic approach in the past have stemmed from a failure to identify mutants with clear, reproducible

or heritable phenotypes - so we consider simplicity to be a prime requisite for a successful screen.

Additionally, a screen for *Arabidopsis* mutants with enhanced susceptibility to UV-B induced leaf damage would complement investigations already in progress which examine other *Arabidopsis* UV-B responses (eg. inhibition of hypocotyl elongation, UV-B induced phototropism and *CHS* expression).

Conversely, a major disadvantage of undertaking the proposed screen is that it is not tailored specifically to isolating mutants altered in UV-B perception and signalling. In addition to UV-B perception / signalling mutants, which are of the greatest interest to us, a screen for mutants which are hypersensitive to UV-B induced leaf damage may also yield mutants deficient in DNA repair enzymes, antioxidant systems or phenylpropanoid biosynthesis. Consequently, it would be necessary to further examine putative mutants to identify those which were altered in UV-B signalling or perception, once the initial screen had been completed.

Nevertheless, separating UV-B signal transduction mutants from less interesting ones should not be an intractable problem. For example, many phenylpropanoid biosynthetic enzyme mutants are readily identifiable by their loss of seed coat pigment ('transparent testa') and mutants arising from the proposed screen which are altered in their capacity for DNA repair might be identified by cross pollinating these with known DNA repair mutants.

It seems likely that the problems inherent in screening for mutants with enhanced sensitivity to UV-B induced leaf damage for our purposes, do not outweigh the benefits of the simplicity of the screen and its potential for generating interesting mutants which could be subsequently characterized.

We further considered the possibility of screening for increased susceptibility to supplementary UV-B induced leaf damage in an M_2 population containing our *CHS-Luc* reporter gene, as this would provide an additional method of identifying mutants likely to be of interest (i.e. those with reduced levels of UV-B induced *CHS* gene expression). We could adjust the screening conditions so that mutants sensitive to tissue damage can be rescued,

allowing us to focus on positive regulators of UV-B damage resistance in the hope of getting mutants deficient in UV-B perception or signalling.

3.6.3 Pilot Experiments Demonstrated that 12 Day Old Mutant Plants Treated with High Fluence Rate UV-B Supplemented with White Light Could Be Distinguished from wild-type Plants by their Increased Susceptibility to UV-B Induced Leaf Damage and that These Mutants Could Subsequently be Rescued

Three pilot experiments were carried out using various different supplementary UV-B treatment durations and the results of selected treatment durations, from pilot experiments 2 and 3, are shown together in Figure 3.4. The spectra shown previously in Figure 3.2 were actually generated during the third pilot experiment.

L. *er* and Col3 wild-type, together with *chum31*, *uvr8*, *fah1*, *tt4*, *tt5* and *30e5bc2* mutant plants were grown on compost, in seed tray inserts, under approximately 120 $\mu Em^{-2}s^{-1}$ white light for 12 days before UV-B was applied. Plants were spaced out to a density appropriate for illumination. Seed tray inserts were then treated with 5 $\mu Em^{-2}s^{-1}$ supplementary UV-B (with approximately 40 $\mu Em^{-2}s^{-1}$ white light) for 0, 4, 12, 18, 24, 30 and 36 hours respectively before returning to 120 $\mu Em^{-2}s^{-1}$ white light for recovery. A fresh cellulose acetate filter (changed every 24 hours) was used to eliminate UV-C wavelengths. One insert was treated for 30 hours under identical conditions except that a filter was applied to remove the UV-B part of the spectrum only. The latter set-up is a 'UV-B Minus' control, the result from which is also shown in Figure 3.4, demonstrating that damage to plants is produced by the UV-B wavelengths applied to the other seed tray inserts but not the control. Photographs taken to demonstrate the effect of UV-B immediately after and also several days after each treatment are shown (Figure 3.4).

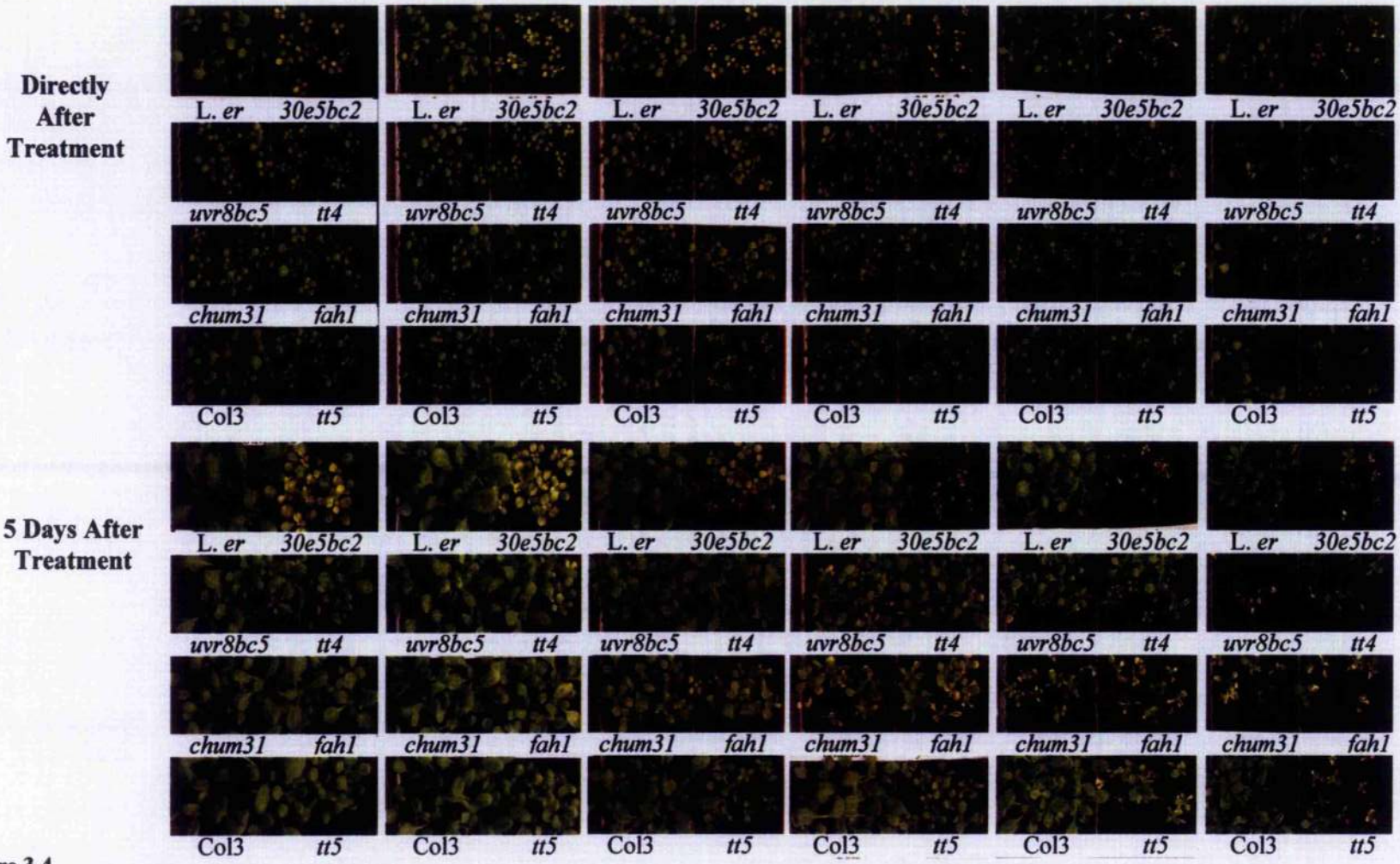


Figure 3.4

Sensitivity of *Arabidopsis thaliana* to Supplementary UV-B Induced Damage. Wild-type and mutant *Arabidopsis thaliana* were grown under 120 $\mu Em^{-2}s^{-1}$ white light for 12 days before treatments of varying durations (Left to right: 0 hrs; UV-B Minus (30 hrs); 18 hrs; 24 hrs; 30 hrs; 36 hrs) with 5 $\mu Em^{-2}s^{-1}$ UV-B (Supplemented with 40 $\mu Em^{-2}s^{-1}$ white light) and then returned to 120 $\mu Em^{-2}s^{-1}$ white light for a 5 day recovery period.

The results shown in Figure 3.4 clearly demonstrate that some mutants (for example *fah1*) are more susceptible to UV-B induced damage than others (for example *tt4*). Nonetheless, from the perspective of a screen which we may yet decide to initiate, it seems reasonable to conclude that 24 hours of 5 $\mu Em^{-2}s^{-1}$ supplementary UV-B (with 40 $\mu Em^{-2}s^{-1}$ white light) applied to 12 day old (120 $\mu Em^{-2}s^{-1}$ white light grown) plants will produce obvious damage in many potentially interesting mutants whilst minimizing lethality. Putative mutants isolated from a screen based on these conditions could furthermore be allowed to recover in white light of reduced fluence rate (eg. 50 $\mu Em^{-2}s^{-1}$), perhaps enhancing their chances of survival.

3.7 Discussion

3.7.1 Plant Responses, Specifically to UV-B Wavelengths of Light, were Sought to Form the Bases of Screens Designed to Isolate Mutants Lacking Components Involved in UV-B Perception or Signal Transduction

The present volume of work, in its entirety, has a single underlying objective and that objective is to identify some of the cellular components which allow *Arabidopsis* to detect UV-B or carry the signal(s) from UV-B perception to effect characteristic responses.

The challenge of unravelling UV-B perception and signal transduction pathways in *Arabidopsis* is heightened by the fact that the plant has a number of responses to UV-B and more than one perception / signal transduction pathway. The complex background to UV-B responses in higher plants and the state of the literature in the field has already been described in detail in Chapter One and need not be reiterated here. However, UV-B appears to be capable of eliciting at least two very different *types* of response in *Arabidopsis*, and so it should be remembered that current evidence suggests low fluence rate UV-B may induce photomorphogenesis by a photoreception / signal transduction pathway, whereas high fluence rate UV-B may generate tissue / biomolecular lesions leading to various physiological processes designed to minimize lasting damage to the plant. It is not clear how UV-B stimulated phenylpropanoid biosynthesis, and in particular *CHS* gene expression, integrates within the complex network of UV-B induced cell signalling pathways (see Chapter One).

In order to develop screens for mutants deficient in UV-B induced responses we first had to identify a number of candidate responses and confirm these were indeed induced specifically by UV-B (280-320 nm) light. Demonstrating that responses under investigation were dependent upon UV-B wavelengths was complicated by the fact that our UV-B 313 fluorescent tubes also emit UV-A, blue, green and far-red light (as Figure 3.1 confirms). Very low fluence rates of light may be capable of inducing photomorphogenesis for example, and so the non-UV-B wavelengths produced by our fluorescent tubes are problematic insofar as these other light qualities might stimulate responses such as the inhibition of hypocotyl

elongation, potentially masking the effect of UV-B. Nevertheless, it was established early in the present work that trying to undertake mutant screens with monochromatic UV-B light sources would be impractical.

Therefore, to ensure that the responses under study were reliant upon UV-B wavelengths, we employed a filter, designated 'clear 130' (Lee Filters, Andover), which fails to transmit only the UV-B part of the spectrum. Responses of *Arabidopsis* which are dependent on UV-B wavelengths should be abolished by the application of the 'clear 130' filter to our UV-B 313 tubes. Indeed, the same filter had previously been used to demonstrate that *CHS* expression in *Arabidopsis* cell culture was produced by UV-B wavelengths from our fluorescent tubes (Christie and Jenkins, 1996). Additionally, one notable advantage of using a filter which highlights the difference between a plant responding and one not responding to UV-B, is that phenotypes exhibited by *Arabidopsis* under the 'UV-B Minus' treatment (ie. lacking the UV-B wavelengths) may reflect phenotypes of the mutants we hope to isolate.

There is another very important practical constraint impinging on any screen for *Arabidopsis* mutants deficient in UV-B responses: plastic Petri dish lids covering the agar plates on which seedlings are often grown as an alternative to compost significantly impair the transmission of UV-B light. Removing the lids before UV-B treatments are applied is not a solution to the problem because the agar will dry from the surface of an uncovered plate completely within about 24 hours. Therefore, in the work described herein plants were either grown on compost before UV-B irradiation, or fluence rate measurements were made through a Petri dish lid to ensure that the actual dosage of UV-B received by seedlings was accurately recorded.

Once responses have been identified which depend upon UV-B wavelengths, screens can be initiated for mutants which lack these particular UV-B responses in the hope of ultimately identifying cellular components needed for UV-B perception or signalling in *Arabidopsis*.

3.7.2 Four Responses of *Arabidopsis* to UV-B (Inhibition of Hypocotyl Elongation, Phototropism, Leaf Damage and *CHS* Expression) were Investigated for their Suitability as the Bases of Screens to Isolate UV-B Perception / Signalling Mutants

Four responses of *Arabidopsis* to UV-B, two in etiolated seedlings (inhibition of hypocotyl elongation and UV-B induced phototropism) and two in light grown, mature leaf tissue (*CHS* gene expression and supplementary UV-B induced leaf damage) were examined with a view to assessing their suitability as bases for initiating mutant screens. For the two responses under study in seedlings, mutant backgrounds (especially *cry1cry2* for the inhibition of hypocotyl elongation response and *phot1phot2* for UV-B induced phototropism) were investigated, in addition to wild-type seedlings, to examine the capacity UV-B has for inducing each response without the interference of some of the major blue light photoreceptors.

The four responses under study were not observed when the seedlings / plants were in the untreated / pre-treated state. Conversely, in each case a very strong response was observed once UV-B illumination was applied: strong inhibition of hypocotyl elongation, strong phototropic curvature towards the light source, extensive leaf damage plus growth inhibition and strong *CHS* gene expression, respectively. The crucial test however, involved ensuring that each response was abolished when the 'clear 130' filter, which prevents illumination with UV-B wavelengths, was applied to the UV-B fluorescent tubes. Comparing the results of a 30 hour supplementary UV-B treatment with those of a treatment of similar duration lacking the UV-B wavelengths ('UV-B Minus'), seen in Figure 3.4, provides a clear indication that UV-B wavelengths are required to induce the damage to plants shown. Similarly, we can be confident that it is UV-B wavelengths from the UV-B fluorescent tubes which are critical in inducing *CHS* gene expression in mature leaf. The latter conclusion was reached by Christie and Jenkins (1996) during their studies with *Arabidopsis* cell culture, and the lack of any significant induction of *CHS* gene expression by low fluence rate UV-A / blue or red / far-red light in mature leaf tissue has been demonstrated directly (Christie and Jenkins, 1996, Wade et al., 2001, Jenkins et al., 2001). Since 'UV-B Minus' treatment has essentially no effect in

producing UV-B induced leaf damage or *CHS* expression respectively, these clear responses are entirely dependent on UV-B wavelengths from the fluorescent tubes and from this perspective would form ideal bases for mutant screens.

By contrast, the UV-B responses studied in etiolated seedlings proved to be less straightforward. In the case of UV-B induced inhibition of hypocotyl elongation it was found, using various backgrounds, that almost the entire ('UV-B Plus') response could be produced even when the UV-B wavelengths were completely removed (ie. virtually the same response could also be generated by 'UV-B Minus' treatment). There did appear to be a reproducible increase in the extent of specifically UV-B induced inhibition of hypocotyl elongation in the *cry1cry2* double mutant, but the increase was small and there was some evidence that non-UV-B wavelengths played the greater part in stimulating the overall response, even in this *cry1cry2* mutant. It should also be noted that phytochromes could play a direct role in mediating UV-B induced inhibition of hypocotyl elongation in *Arabidopsis thaliana* seedlings (Kim et al., 1998).

Finally, the phototropic response of *phot1phot2* double mutant seedlings to UV-B also presented a problem: In the dark (ie. before treatment) all seedlings grew directly upwards and when UV-B of appropriate fluence rate was applied most turned strongly toward the light source. However, when the UV-B wavelengths were removed (ie. 'UV-B Minus' treatment was applied), most of the seedlings turned in one direction or the other but the consistent trend for strong curvature towards the light source was lost. An explanation for this 'random hypocotyl-bending' was provided by Ohgishi *et. al.* (2004) who used blue light to demonstrate that the cry1 and cry2 photoreceptors can mediate random curvature in the absence of the phototropin photoreceptors.

Thus, it is clear from the present studies that the UV-B responses investigated in *Arabidopsis* seedlings (inhibition of hypocotyl elongation and UV-B induced phototropism) do not form ideal bases for mutant screens as, in each case, 'UV-B Minus' treatment elicits some kind of response.

3.7.3 Decisions were made to Proceed with Two Screens: One for Mutants Altered in UV-B Induced *CHS* Expression and the Other for Mutants Lacking a UV-B Induced Positive Phototropic Hypocotyl Response

A screen for mutants which lack (or are altered in) the ability to induce *CHS* expression in response to UV-B was initiated. Because UV-B strongly induces *CHS* expression *via* a signal transduction pathway which does not require known UV-A / blue photoreceptors (such as the cryptochromes) we believed that a mutant screen based on this UV-B response afforded a good chance of success. A detailed account of the screen and its results is provided in Chapter Four.

By contrast, we decided that it would be unwise to initiate a screen for mutants which lacked UV-B induced inhibition of hypocotyl elongation. Our pilot experiments had demonstrated a UV-B specific increase in hypocotyl growth inhibition using the *cry1cry2* double mutant background. However although reproducible, the observed response was small and it was clear from studies in the wild-type, other mutant backgrounds and possibly even the *cry1cry2* background that non-UV-B wavelengths generated by our UV-B fluorescent tubes were responsible for the greater part of the inhibition of hypocotyl elongation response. Initiating a screen under such circumstances would be unwise because resulting mutants could be altered in their response to non-UV-B wavelengths of light. We surmise that the success enjoyed by other investigators employing similar screens (Suesslin and Frohnmeyer, 2003) has depended on the use of different UV-B fluorescent tubes and / or experimental conditions from those we used in our studies.

Assessing the likelihood of success in screening for mutants which lacked UV-B induced positive phototropic hypocotyl curvature was a difficult exercise. By employing *phot1phot2* in our pilot experiments and preparing to screen using the double mutant background, we knew that neither phototropin could be isolated in our screen nor could they interfere with any phototropic response which might be elicited by UV-B. However, the pilot experiments highlighted a response to non-UV-B wavelengths (ie. 'UV-B Minus' treatment) and that response was random hypocotyl-bending which is mediated by the cryptochromes (Ohgishi et

al., 2004). Ideally, responses to wavelengths other than UV-B should not be allowed to interfere with our proposed screens at all. Nonetheless, since strong hypocotyl curvature towards the light source seemed to be a consistent trend which depended specifically on UV-B wavelengths, we decided to initiate a screen for mutants which lacked UV-B specific curvature using the *phot1phot2* double mutant background. A detailed account of the development and results of the screen is provided in Chapter Five.

It is clear that plants display a variety of responses to UV-B. In our studies on inhibition of hypocotyl elongation, phototropism and *CHS* gene expression it seemed that UV-B was acting as a signal for the production of each response. However, in studying supplementary UV-B induced leaf damage and growth inhibition in *Arabidopsis* it appeared that we were not looking directly for the loss of a UV-B signalling response but rather for increased tissue damage caused by UV-B. Such damage is probably primarily a physical, rather than a biological, response although damage can induce signalling and *vice versa*. From a selective viewpoint, it is unsurprising that *Arabidopsis* may induce *CHS* expression and flavonoid biosynthesis in response to UV-B to protect itself from subsequent UV-B induced tissue damage. The fact that there is a link between the two aforementioned UV-B responses (see Chapter Four) suggests that it may yet be worthwhile initiating a screen for mutants with an increased susceptibility to supplementary UV-B induced leaf damage as a means of understanding UV-B signalling in *Arabidopsis*, although it was decided not to initiate such a screen as part of the present work. Nevertheless, Chapter Six does contain a description of the characterization of a mutant called *30e5* which undoubtedly shows hypersensitivity to UV-B induced leaf damage.

CHAPTER 4

MUTANTS ALTERED IN ULTRAVIOLET-B INDUCED EXPRESSION OF THE *Arabidopsis CHALCONE SYNTHASE* GENE

4.1 Introduction

Plants originated in an aquatic environment and pivotal for their successful adaptation to a terrestrial existence was a capacity for generating large quantities of phenolic compounds, many of which are of structural importance. Most plant phenolics are products of phenylpropanoid metabolism and these have adopted a wide range of physiological roles. One subgroup, the flavonoids, well illustrates the functional diversity attributable to plant phenolics (Buchanan et al., 2000). Nearly 5000 flavonoids have been identified thus far including anthocyanin pigments (which recruit pollinators and seed dispersers), molecules involved in plant-microbe interactions and male fertility, antimicrobial agents, feeding deterrents and epidermally located UV-protectants. As a result of this multiplicity of roles, the first committed step in flavonoid biosynthesis constitutes an important control point in plant secondary metabolism and that particular reaction is catalyzed by the enzyme chalcone synthase (CHS). Expression of the *CHS* gene, which is stimulated by light and various other environmental and endogenous stimuli, is a focal point of the present work (Buchanan et al., 2000, Winkel-Shirley, 2001, Jenkins et al., 2001).

As described in Chapter One, expression of the single *Arabidopsis CHS* gene is controlled principally at the level of transcription and is stimulated by UV-A / blue light *via* the cryptochrome photoreceptors. Chalcone synthase gene expression in *Arabidopsis* is also strongly induced by UV-B illumination and the system responsible for UV-B photoperception, which has hitherto eluded investigators, is known to be distinct from that involved in UV-A / blue light induced *CHS* expression (Wade et al., 2001). The possibility that there may be a UV-B photoreceptor which is capable of stimulating the flavonoid

biosynthetic pathway was discussed in Chapter One (Section 1.3.3.4.4). Significantly, both the UV-A / blue and UV-B *CHS* induction pathways in *Arabidopsis* appear to converge on the bZIP transcription factor HY5 (Jenkins et al., 2001). HY5 is known to regulate other UV-B stimulated genes (Ulm et al., 2004) and mediates a range of photomorphogenic responses in *Arabidopsis* (Ang and Deng, 1994, Osterlund et al., 2000). Uncovering the mechanism by which higher plants detect UV-B *en route* to responses such as the induction of *CHS* gene expression is currently a matter of no small importance in plant photobiology. Consequently, a genetic approach was used to try to identify UV-B perception and signalling components.

In the work described presently, a transgenic screening method was adopted in order to isolate mutants altered in the regulation of *CHS* gene expression. One of the main reasons that control of *CHS* transcription was chosen as the focus of this screen for mutants altered in UV-B signal transduction was that a number of the previously identified UV-B signalling components additionally known to be involved in wounding or anti-pathogen defence have been shown not to be involved in UV-B induced *CHS* expression (Jenkins et al., 2001). The likelihood of finding novel UV-B signalling components therefore appeared to be high. Several mutants deficient in UV-B induced *CHS* transcript accumulation were discovered, corresponding to changes in a gene called *UVR8*. UVR8 is involved in regulating UV-B responses in *Arabidopsis* (Kliebenstein et al., 2002). Compellingly, it was shown that despite the loss of UV-B stimulated gene expression other signalling routes leading to *CHS* induction (such as the cry1 / UV-A, phyA / far-red and low temperature pathways) remained in the *uvr8* mutant. These data suggest that in the mutant, a signalling pathway specifically induced by UV-B photoperception has been interrupted. Genome-wide transcriptome analysis revealed that the *uvr8* mutant shows a reduced level of UV-B induced expression of a number of flavonoid biosynthetic genes together with several other genes concerned with UV-B protection. Significantly, a reduction in the quantity of *HY5* mRNA generated by UV-B in mature *uvr8* leaf tissue was also observed and confirmed by RT-PCR. The implication of this finding is that UVR8 precedes HY5 in the perception / transduction of a UV-B signal. In accordance with this hypothesis, the *uvr8* and *hy5* mutants showed a similar level of increase

in susceptibility to supplementary UV-B induced growth inhibition and leaf damage. The findings indicate that UVR8 is inextricably linked to the initiation of a key mechanism of UV-B photoreception in *Arabidopsis thaliana*.

4.2 Mutants With Alterations in UV-B Induced *CHS* Gene Expression were Sought Using a *CHS*-Luciferase Reporter Gene Based Screen

4.2.1 We Screened 14 day old Light Grown M_2 *Arabidopsis*, Mutagenized from the *CHS-Luc 3.4* Line, for Altered *CHS* Expression after a 3-4 Hour 3 $\mu Em^{-2}s^{-1}$ UV-B Treatment

4.2.1.1 Establishing Conditions for the Screen

Two transgenic *Arabidopsis* lines had previously been produced by transforming Landsberg *erecta* plants with a chimaeric gene consisting of the *Arabidopsis* chalcone synthase promoter fused to the luciferase coding sequence (Shenton and Jenkins, unpublished work). Each of the two lines (*CHS-Luc 1.7* and *CHS-Luc 3.4*) had been found to have incorporated a single copy of the reporter transgene (Littlejohn and Jenkins, unpublished work) but *CHS-Luc 3.4* was chosen to form the basis of the mutant screen described herein because this line showed the greater level of UV-B induced luciferase production (Figure 4.1).

Testing large numbers of small seedlings at the earliest opportunity may enhance the efficacy of the screen and so it was decided that two week old seedlings should be checked for UV-B induced reporter gene activity. At this stage, seedlings grown under relatively low fluence rate white light will be acquiring true leaves.

It was also decided, based on previous work (Wade et al., 2003) and the timecourse data shown in Figure 4.1, that seedlings should be treated with approximately 3-4 hours of 3 $\mu Em^{-2}s^{-1}$ UV-B during the screen as significant levels of *CHS* expression and *CHS* promoter driven luciferase production should result.

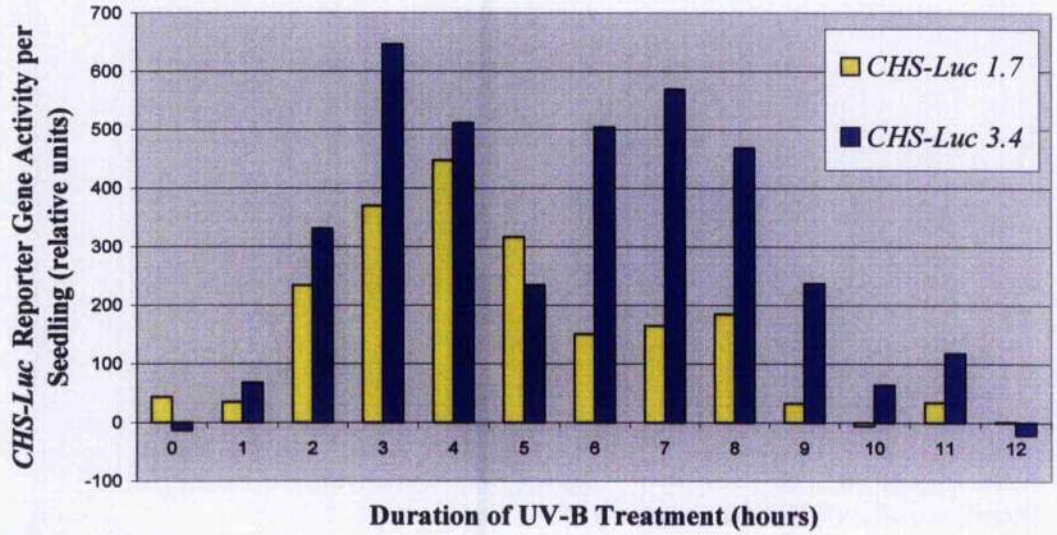


Figure 4.1
CHS-Luc Screen: Preliminary Timecourse. A high level of *CHS* promoter driven luciferase activity is observed after 3-4 hours of 3 $\mu Em^{-2}s^{-1}$ UV-B treatment, especially in the *CHS-Luc 3.4* line. Therefore this treatment duration and line were utilized in the screen. Seedlings were grown on compost under non-inducing conditions (approximately 25 $\mu Em^{-2}s^{-1}$ white light) for 15 days before 3 $\mu Em^{-2}s^{-1}$ UV-B treatments were provided for the durations shown. Photon counting imaging was performed and *CHS* promoter activity, based on the UV-B induced increase in luminescence, calculated per seedling in relative units (see Materials and Methods - Section 2.9.2.2).

4.2.1.2 The Luciferase Stage of the Screen Produced 129 Putative Mutants Apparently Altered in UV-B Induced *CHS* Expression

Mutageneses were performed on approximately 147,000 *CHS-Luc 3.4* seeds in total using differing concentrations of Ethyl Methane Sulfonate (EMS). Approximately 7,000 M_1 plants were produced and allowed to self-pollinate and the resulting M_2 seed was separated into 224 batches, each batch containing seed from about 20-40 M_1 plants. Approximately 52,000 *CHS-Luc 3.4* M_2 seedlings in total were screened, so approximately 7.4 M_2 seedlings were screened per M_1 plant. Since a minimum of 4 M_2s should be screened for each M_1 plant (due to the fact that many mutations are recessive) this figure of 7.4 suggests the screen should be successful in identifying mutants generated – although some M_2 batches were examined more exhaustively than others.

The M_2 seedlings were screened for significantly reduced or increased *CHS* promoter activity using a photon counting camera and the introduced luciferase reporter gene. A large number of putative mutants (approximately 1,100) were isolated from the first stage of the screen (an image depicting UV-B induced luciferase expression in 'Stage One' M_2 seedlings can be seen in Figure 4.2). Several seeds from each putative mutant selected were sown in wells of seedling packed insert trays and re-screened in a second stage (see image shown in Figure 4.3), to ascertain which of the putative mutants were consistently altered in UV-B stimulation of *CHS-Luc*. Sometimes a third stage of screening was used to examine putative mutants which were ambiguous. Luciferase expression level comparisons were simply made between neighbouring seedlings. To illustrate a contrast, an image of *CHS-Luc 3.4* seedlings taken under non *CHS* inducing conditions (low white light) is shown in Figure 4.4 along with a corresponding image of the same seedlings after 4 hours of 3 $\mu Em^{-2}s^{-1}$ UV-B. Putative mutants which still appeared to be low expressors of *CHS-Luc* after the second or third stages of screening were designated *chum* (*CHS* underexpressing mutants) and overexpressors were called *chom* mutants. A few *chum* / *chom* putative mutants were chosen directly from the first stage of the screen (ie. without being checked at the second stage).

Finally, a total of 129 putative mutants (113 *chums* and 16 *choms*) emerged from the luciferase phase of the screen and these were all set aside to be checked by RT-PCR for corresponding alterations in endogenous *CHS* expression.

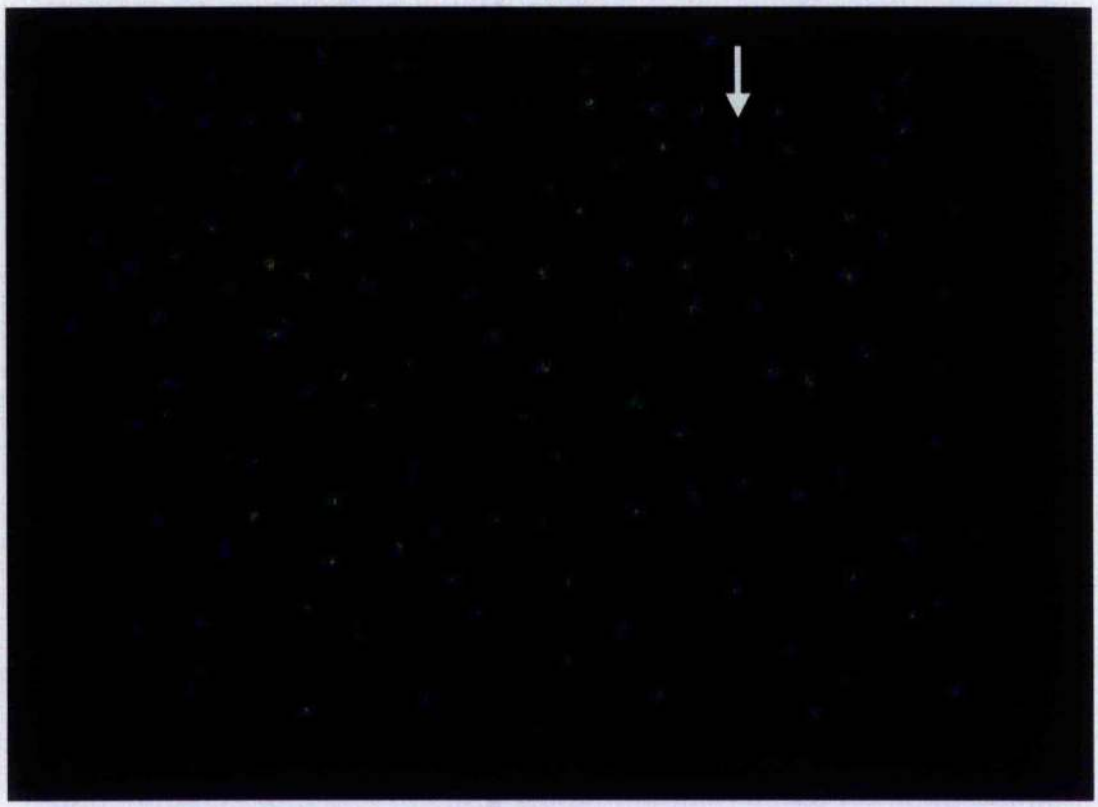


Figure 4.2
CHS-Luc Screen: Stage One. Image generated using a photon counting camera of 14 day old *CHS-Luc 3.4* M_2 seedlings grown under approximately 30 $\mu Em^{-2}s^{-1}$ white light and then treated with 3-4 hours of 3 $\mu Em^{-2}s^{-1}$ UV-B to induce *CHS* promoter activity (see Materials and Methods). Putative low expressing mutants, such as the seedling arrowed, were isolated and the resulting M_3 progeny tested again for Luciferase activity.

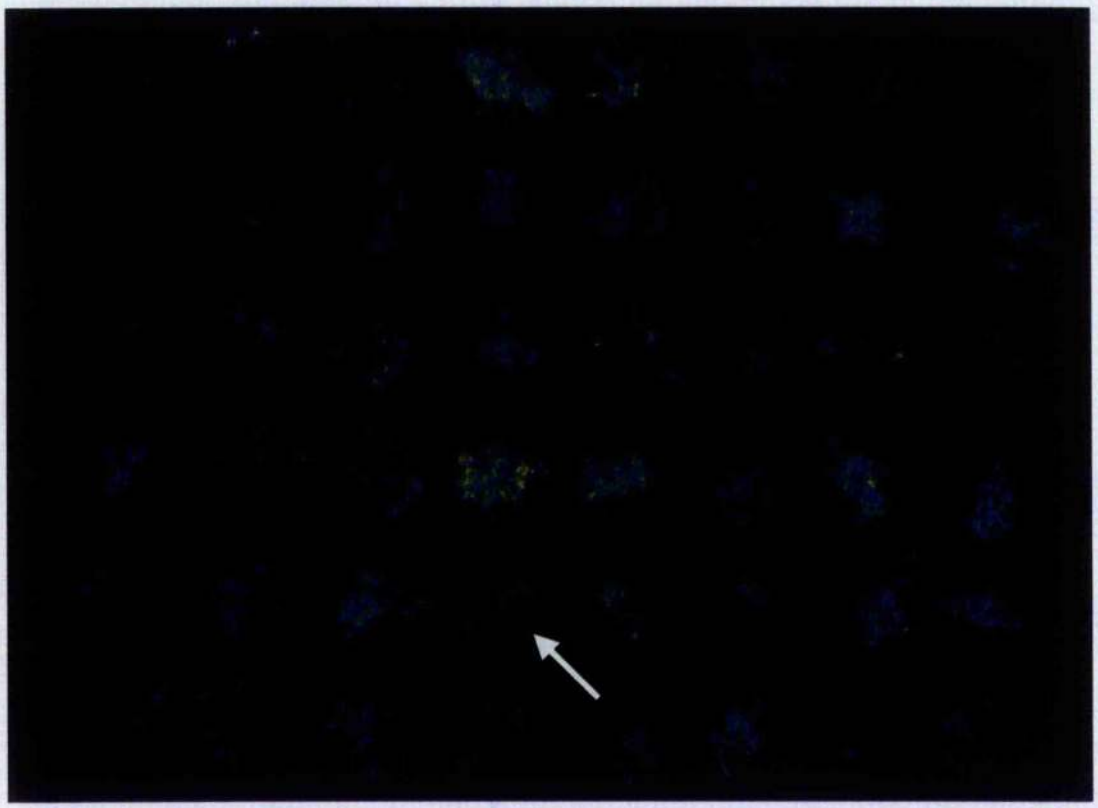


Figure 4.3
CHS-Luc Screen: Stage Two. Image generated using a photon counting camera of 14 day old *CHS-Luc 3.4* M_3 seedlings grown under approximately 30 $\mu Em^{-2}s^{-1}$ white light and then treated with 3-4 hours of 3 $\mu Em^{-2}s^{-1}$ UV-B to induce *CHS* promoter activity (see Materials and Methods). Here groups of M_3 seedlings, each derived from a single M_2 putative mutant, are grown up together in a tray. Putative low expressing mutants, such as the group of seedlings arrowed, were selected to test for reduced levels of UV-B induced endogenous *CHS* gene expression.

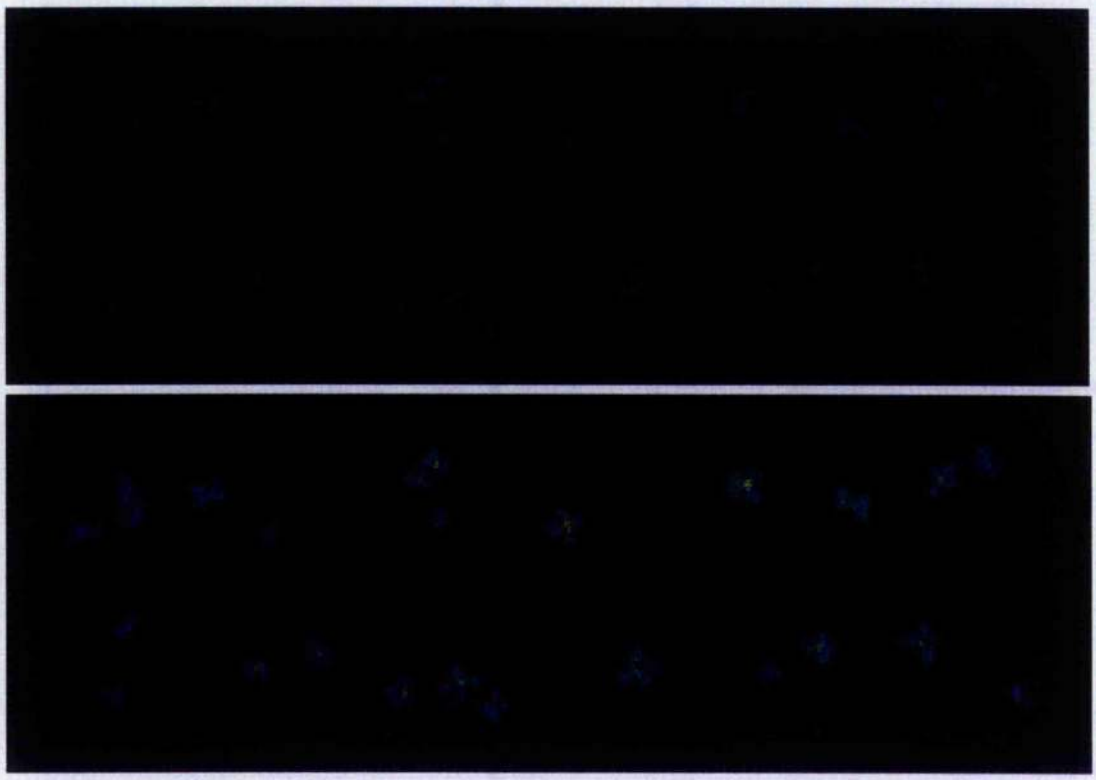


Figure 4.4
The Effect of UV-B on *CHS-Luc*. Juxtaposition of untreated (above) and UV-B treated (below) seedlings under conditions similar to those applied during the *CHS-Luc* screen. Images were generated using a photon counting camera of *CHS-Luc 3.4* seedlings grown under approximately 25 $\mu Em^{-2}s^{-1}$ white light for 15 days before (above) and after (below) a 4 hour treatment of 3 $\mu Em^{-2}s^{-1}$ UV-B was applied. The upper image illustrates that under non-inducing conditions, ie. before UV-B treatment, very little *CHS* promoter driven luciferase production is observed. These images supplied part of the data used to generate Figure 4.1.

4.3 Endogenous Levels of *CHS* Expression in Each Putative Mutant were Examined by Quantitative RT-PCR

4.3.1 *ACTIN2* and *CHS* Primers Were Combined in a Single Reaction Tube for 22 Cycles During Quantitative Reverse Transcriptase Polymerase Chain Reaction to Measure *CHS* Expression Levels in the Putative Mutants

Transgenic mutant screens can generate large numbers of putative mutants which must be examined carefully to identify those harbouring mutations of interest. True mutants of UV-B induced *CHS* gene expression will, in addition to transgene activity, be altered in the expression of the endogenous *Arabidopsis CHS* gene - probably because a component involved in carrying the UV-B signal to the *CHS* gene promoter has been damaged by mutagenesis. Rather than use northern blotting to investigate endogenous *CHS* expression levels in each putative mutant, we decided to employ quantitative reverse transcriptase PCR.

It is important to understand that in order to quantify the *CHS* mRNA present in the original RNA samples using quantitative RT-PCR the number of PCR cycles used to amplify the cDNA template must be carefully chosen. Two cDNA / RNA samples can only be reliably compared whilst both remain in the exponential phase of amplification during qRT-PCR. Consequently, after choosing the best *ACTIN2* (Fontaine et al., 2002), loading control, and *CHS* primers to use in the characterization of putative mutants (Figure 4.5), pilot experiments were done to determine optimal conditions for RT-PCR. It was found that approximately 22 cycles of amplification provided visible quantities of PCR product whilst at the same time sampling both *ACTIN2* and *CHS* gene expression during the exponential phase of template amplification in the PCR reaction (Figure 4.6). An additional test experiment was performed to ensure that whether added separately or together in a single reaction tube, the *CHS*1 and *ACTIN*2as primers produced similar results (Figure 4.7).

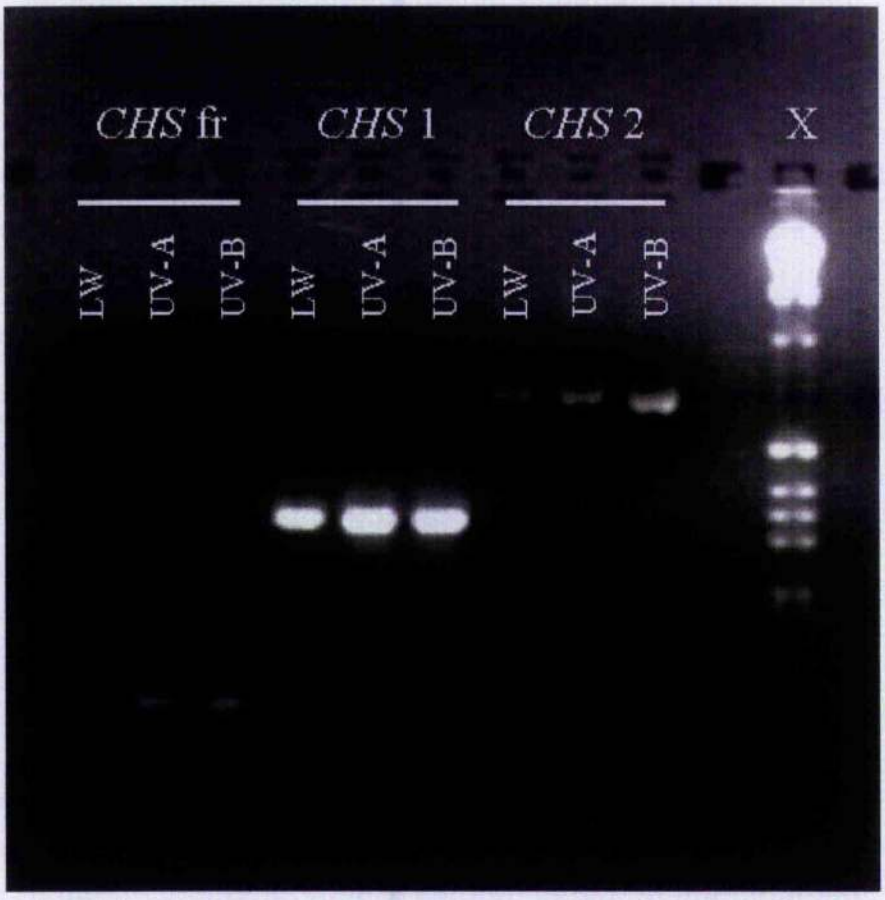


Figure 4.5
Primers which amplify *CHS* cDNA strongly were chosen to characterize the putative mutants. The performance of three pairs of oligonucleotide primers (*CHS*fr, *CHS*1 and *CHS*2) was compared to see which gave the best *CHS* signal (see Materials and Methods). Each of three cDNA samples (LW, UV-A and UV-B) were amplified for 25 cycles of PCR and the resulting products displayed on a 2% agarose gel. The most effective (*CHS*1) primers were chosen for subsequent work. A similar test determined that *ACTIN2as* primers (Fontaine et al., 2002) could be used to confirm that equivalent amounts of total mRNA were represented in each lane.

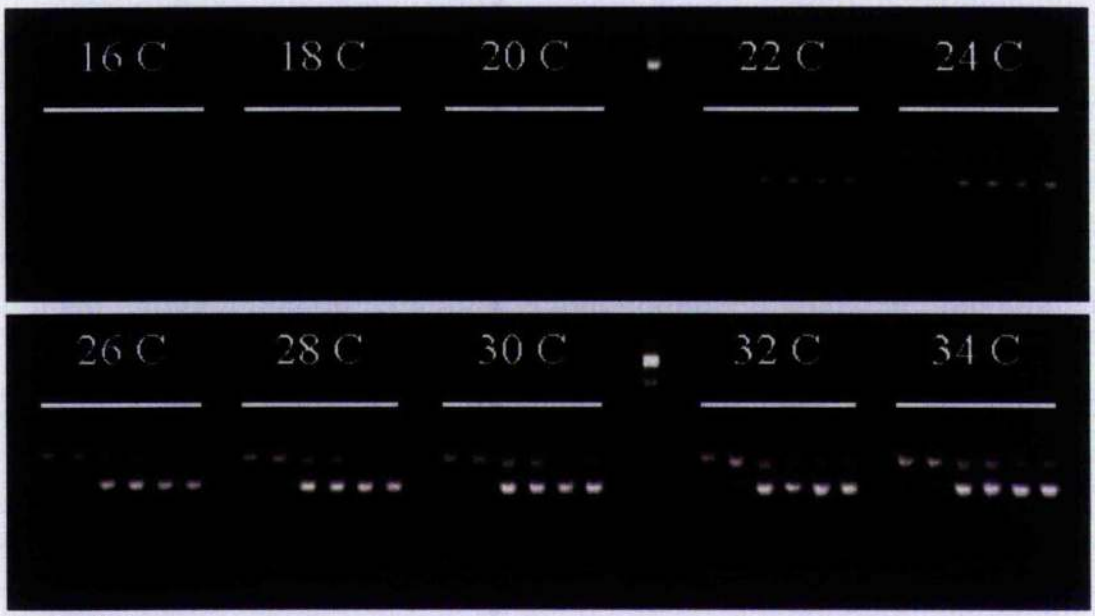


Figure 4.6
22-26 PCR cycles may be used to characterize the putative mutants. *CHS*1 (lower band in each lane at 337 bp) and *ACTIN2as* (the upper band at 500 bp) RT-PCR products accrue as the number of cycles (C) increases. PCR products are clearly visible and appear to be accumulating exponentially at 22 cycles. Results shown are generated from the same three cDNA samples (L. *er* treated with LW, UV-A and UV-B respectively) duplicated at each cycle number.

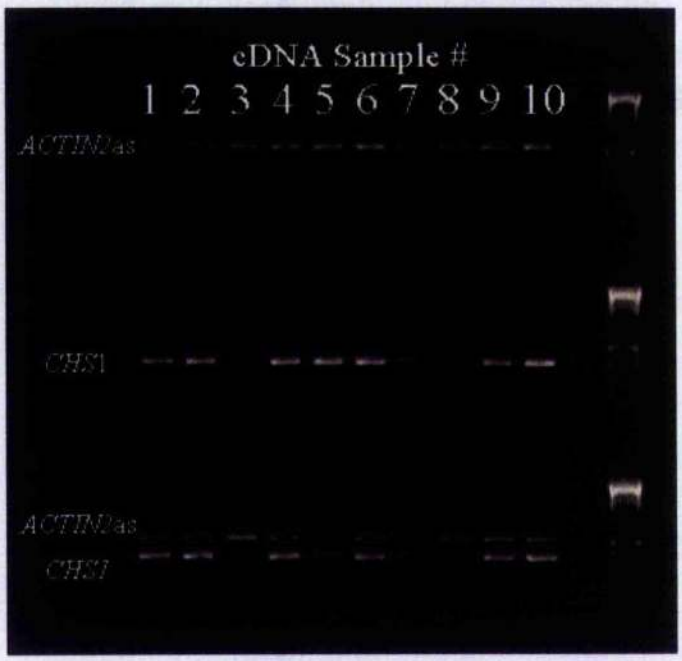


Figure 4.7
The *CHS* and *ACTIN* primers used in the present study provide similar qRT-PCR results when used separately, or together in a single PCR reaction tube. 3 µl of ten separate cDNA samples was amplified for 22 cycles with the two sets of primers added either separately (Top Row - *ACTIN2as*; Middle Row - *CHS1*), or together (Bottom Row) in the same reaction tube. It is not clear why the *ACTIN* control in lane 5 (Bottom Row) is invisible as a master mix was used in this experiment to generate the results.

4.3.2 Endogenous *CHS* Gene Expression Levels Induced by UV-A and by UV-B Light were Quantified by RT-PCR in 3 week old Low White Light Grown Putative Mutants

Clearly 'mutants' which have become altered in the activity of the transgene only are of little interest to us. Conversely, mutants which are altered in UV-B but not UV-A induced endogenous *CHS* gene expression are of particular interest, since these may highlight signalling components specifically concerned with the elusive UV-B signal transduction pathway(s). Therefore, putative mutants were grown under non inducing conditions (approximately 25 $\mu Em^{-2}s^{-1}$ white light) for 3 weeks and *CHS* expression levels subsequently quantified by qRT-PCR (see Materials and Methods) after either no further treatment, 6 hours of 100 $\mu Em^{-2}s^{-1}$ UV-A or 4 hours of 3 $\mu Em^{-2}s^{-1}$ UV-B. All 129 putative mutants (except one which was destroyed by insects before it could bear seed) were examined in this way and a sample of the data is shown in Figure 4.8. Often UV-A and also UV-B treatment of wild-type L. *er* generated *ACTIN* bands of similar intensity to those of the *CHS* signal, facilitating the identification of true mutants from amongst putative mutants.

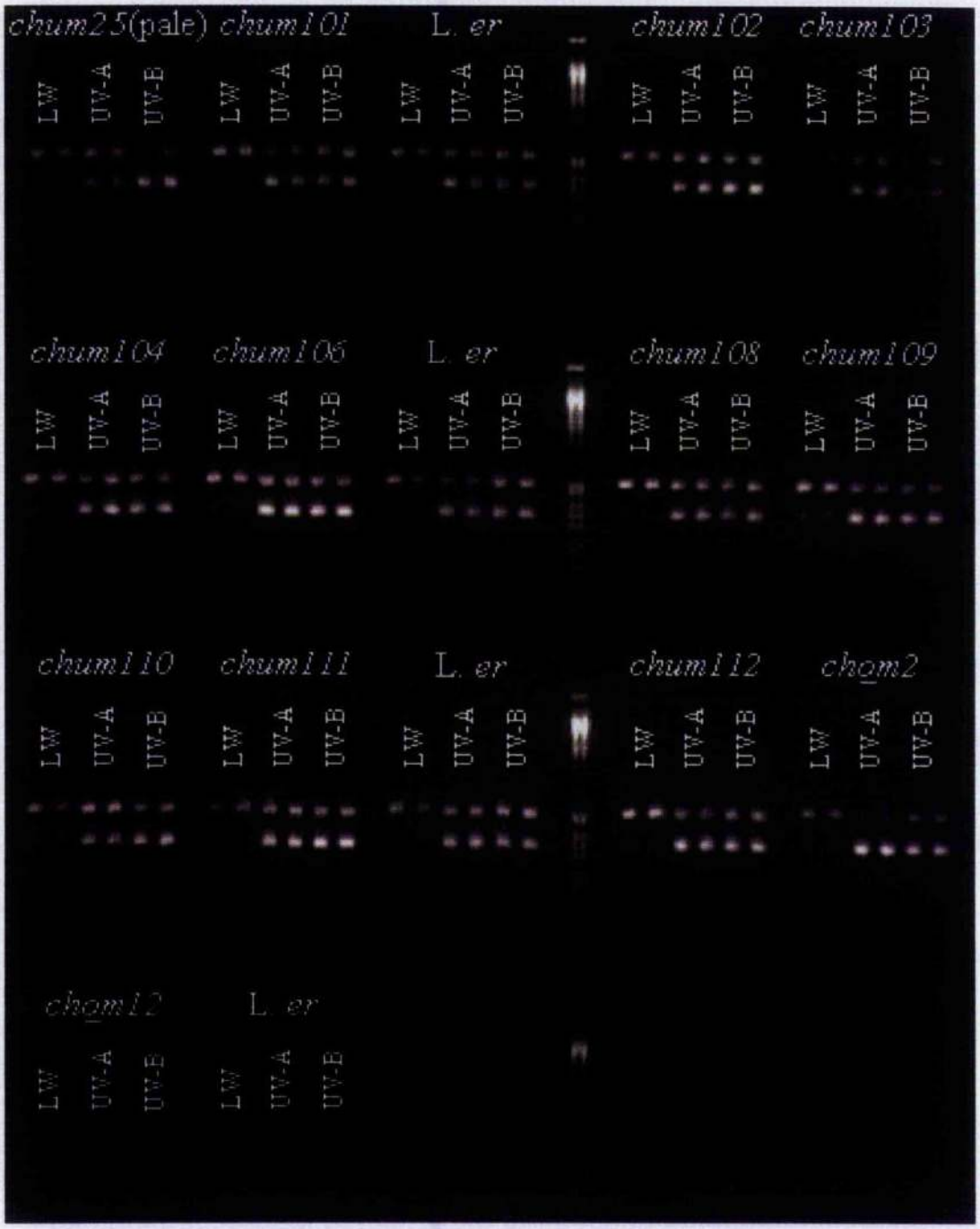


Figure 4.8
Characterization of Putative Mutants (a sample of the data only is shown above). Reverse transcriptase (RT)-PCR analysis of *CHS* expression, induced by UV-A and UV-B. L. *er* wild-type and putative mutants were grown up under non-*CHS* inducing conditions (18-35 $\mu Em^{-2}s^{-1}$ white light) for 3 weeks before treatment with either 100 $\mu Em^{-2}s^{-1}$ UV-A for 6 hours or 3 $\mu Em^{-2}s^{-1}$ UV-B for 4 hours or no further treatment (LW). Tissue was harvested for RNA extraction and ethidium bromide stained products are shown in duplicate highlighting *CHS* expression compared to an *ACTIN* loading control. For each plant the *ACTIN* and *CHS* transcripts are represented by the upper and lower bands respectively. None of the putative mutants shown above displayed a reduction in UV-B induced *CHS* transcript levels compared to wild-type L. *er*. In total, 128 putative mutants were examined in this way.

4.3.3 Six Mutants Repeatedly Proved to Have Altered Levels of Endogenous *CHS* Expression in Response to UV-B Treatment – Four of these had Reduced *CHS* Expression and Two had Increased *CHS* Expression

Endogenous gene expression was studied in the putative mutants and those which appeared to be consistently altered in UV-B induced *CHS* transcript levels identified (Figure 4.9). The great majority of putative mutants showed no clear alteration in *CHS* transcript levels and were therefore either weak alleles or false positives. However, two overexpressors (*chom2* and *chum35*) and four underexpressors (*chum31*, *chum33*, *chum53* and *chum75*) which appeared to be altered in the UV-B regulation of *CHS* transcript levels were isolated. The M_2 batches from which each mutant originated are listed below. It should be noted that because of the overlap in batches screened, it was possible that *chum53* was identical (not simply deficient in an allelic gene) to either *chum31* or *chum33* – but not both.

In addition, each mutant was examined for the presence of a visible phenotype but all closely resembled wild-type L. *er* under the conditions shown (Figure 4.10). Whilst flowering however, *chom2* grew slightly taller and developed more slender stems than did wild-type L. *er*.

Underexpressors

chum31 - from Batch 38-41 Plant 4

chum33 - from Batch 24-27 Plant 4

chum53 - from Batch 14, 24 or 38 Plant 1

chum75 - from 80 mM Batch 10 or 12 Plant 1

Overexpressors

chom2 - from Batch 75 Plant 1

chum35 - from Batch 112 Plant 2

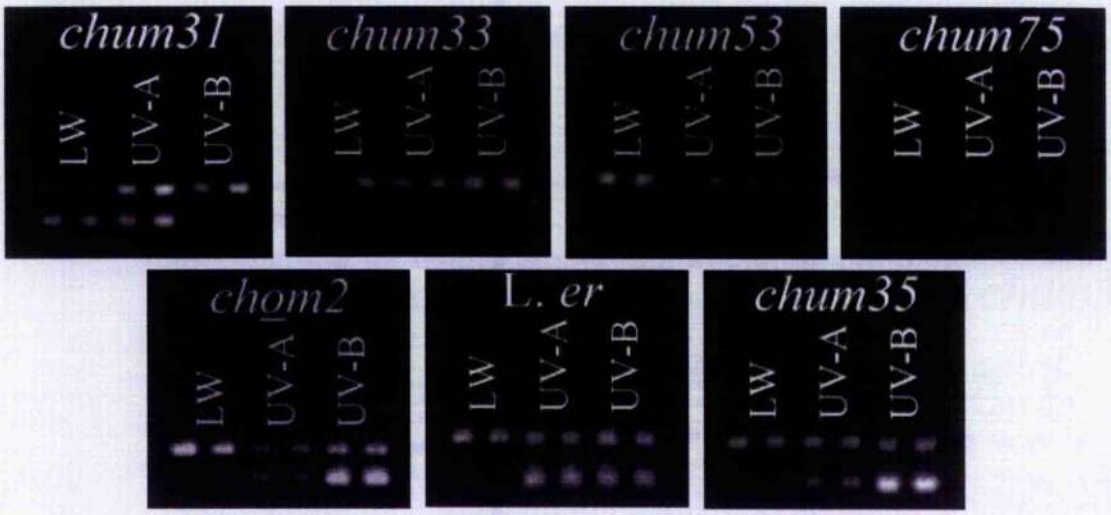


Figure 4.9
Alterations in *CHS* expression for the six mutants. UV-B *CHS* underexpressors are shown on the top row whilst wild-type L. *er* together with the UV-B *CHS* overexpressors are seen on the bottom row. *CHS* expression levels are best judged by comparing the relative intensity of the *CHS* band (lower) with that of the *ACTIN2as* band (upper) in the same lane. Note that LW denotes low white light (ie. RNA / cDNA from control plants which were not provided with a *CHS* inducing light treatment).

Figure 4.10
Visible Phenotypes of each of the six mutants. Wild-type L. *er* and the *CHS-Luc3.4* parent line together with the UV-B *CHS* mutants were grown up under 150 $\mu Em^{-2}s^{-1}$ white light for 16 days.

4.3.4 The Mutations from which All Four UV-B *CHS* Underexpressing Mutants are Derived Appear to be Both Allelic and Recessive

Two overexpressing mutants (*chom2* and *chum35*) were isolated from the screen and may overexpress *CHS* specifically in response to UV-B rather than to UV-A (Figure 4.9). However, it was decided to focus on the *CHS* underexpressors during the present study. Follow-up work on the overexpressing mutants may be conducted at a later date.

To determine whether the same genes were altered in any of the four underexpressing mutants and also to ascertain which mutations were dominant and which recessive, each mutant was crossed to the other three and to wild-type L. *er* respectively. Seed derived from separate crosses (ie. individual siliques) was sown and the F_1 plants grown for 3 weeks under low white light along with the appropriate parent controls. Plants were then treated for 4 hours with 3 $\mu Em^{-2}s^{-1}$ of UV-B and the resulting tissue harvested for RNA extraction and RT-PCR analysis to quantify *CHS* expression levels (Figure 4.11). Comparing *CHS* induction in the F_1s with that in the parents established that all four mutations were both allelic and recessive. It should also be noted that *chum75* appears to be the weakest of the four alleles as a little *CHS* expression occurs in this mutant in response to UV-B (Figures 4.9 and 4.11).

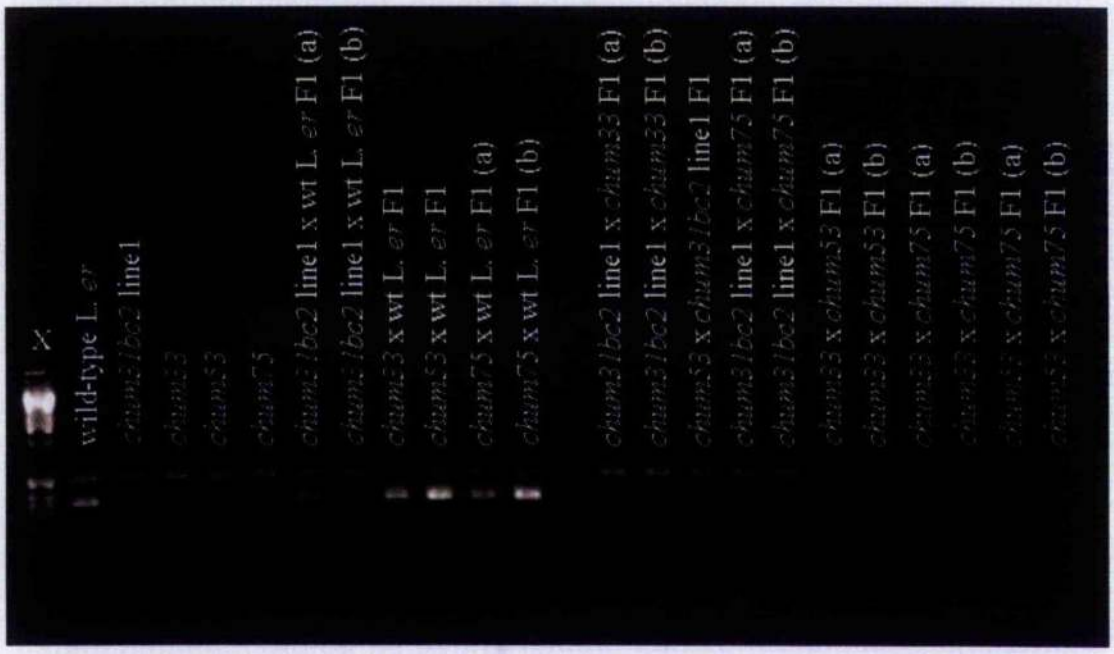


Figure 4.11
Each of the four UV-B *CHS* underexpressors appears to harbour a recessive mutant allele of the same gene. Reverse transcriptase (RT)-PCR analysis of *CHS* mRNA induction by UV-B. F_1 plant tissue from the crosses shown together with the appropriate parents were grown up under non-*CHS* inducing conditions (18-35 $\mu Em^{-2}s^{-1}$ white light) for 3 weeks before treatment with 3 $\mu Em^{-2}s^{-1}$ UV-B for 4 hours. Tissue was then harvested for RNA extraction and ethidium bromide stained products are shown from quantitative RT-PCR depicting *CHS* expression (lower bands) compared to an *ACTIN* loading control (upper bands). *CHS* expression levels are best judged by comparing the relative intensity of the *CHS* band (lower) with that of the *ACTIN2as* band (upper) in the same lane. On the gel above, (a) and (b) indicate (RT)-PCR results from separate crosses. For each cross the female parent is named first. The suffix 'bc2' is an abbreviation for 'backcrossed twice' and denotes a mutant line which has been backcrossed to the wild-type to remove any other mutations (see Section 4.4.3).

Additional evidence that the *chum31* mutation is recessive comes from imaging *chum31* x *CHS-Luc3.4* F_2s for UV-B induction of *CHS* promoter driven luciferase activity (Figure 4.12). Of the 30 F_2 seedlings shown, 24 are thought to be high expressors and only 6 regarded as low expressors. These numbers are certainly consistent with the *chum31* mutation corresponding to a recessive allele.

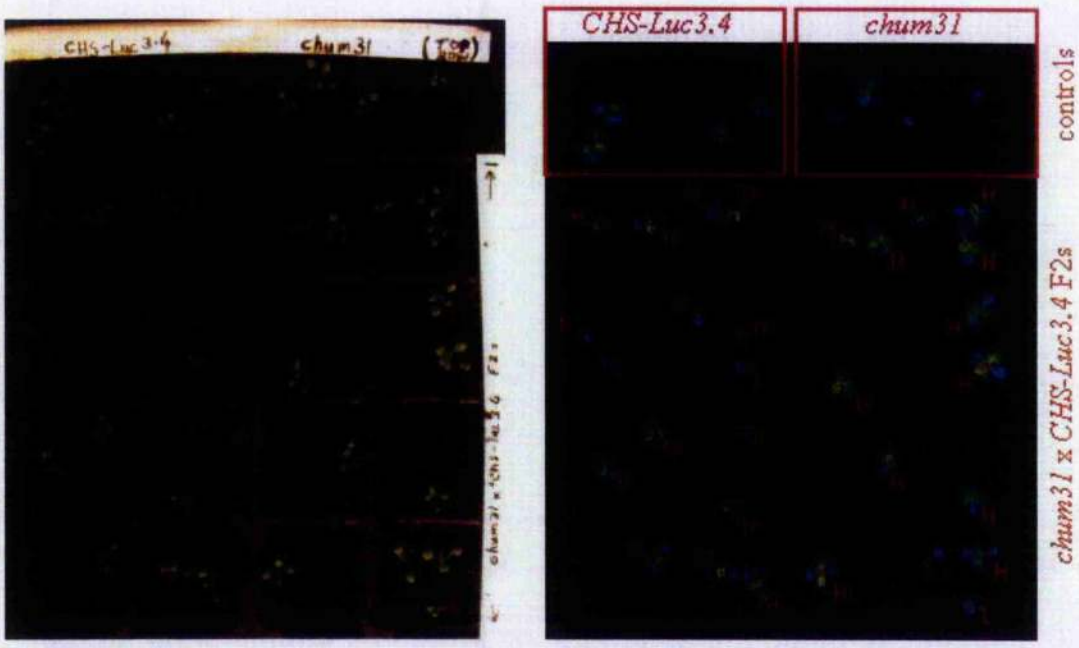


Figure 4.12
Luciferase Imaging of F_2s also demonstrates that *chum31* is a recessive allele. F_2 seedlings from a cross between *chum31* and *CHS-Luc3.4* were grown under 20 $\mu Em^{-2}s^{-1}$ white light for 18 days and then treated for 4 hours with 3 $\mu Em^{-2}s^{-1}$ UV-B to induce luciferase expression. The tray of seedlings is shown (left panel) together with a corresponding image generated using a photon counting camera (right panel). The number of high (H) and low (L) luciferase expressing plants was simply estimated visually by comparison with the parent control seedlings shown along the top row.

4.4 Characterization of the *chum31 / uvr8* Mutant

4.4.1 The *chum31* and *uvr8* Mutants are Altered in Allelic Genes

To test whether a previously discovered mutant might be altered in a gene allelic to that highlighted by our screen, seed from the *uvr8* (L. *er*) mutant (Kliebenstein et al., 2002) was obtained. The *uvr8* mutant shows a significant reduction in UV-B induced *CHS* transcript accumulation and the mutant allele is recessive. The *chum31* and *uvr8* mutants were crossed together and the F_1 progeny treated with UV-B to induce *CHS* gene expression as before (Figure 4.13). *CHS* expression levels in the F_1 plants were found to be reduced in response to UV-B, and reduced to the same extent as in each parent (*uvr8* and *chum31*). Wild-type L. *er* plants, by contrast, show clearly elevated levels of UV-B induced *CHS*. These data indicate that *chum31* and hence the other three recessive alleles described above (*chum33, chum53* and *chum75*) are alleles of the *UVR8* gene.

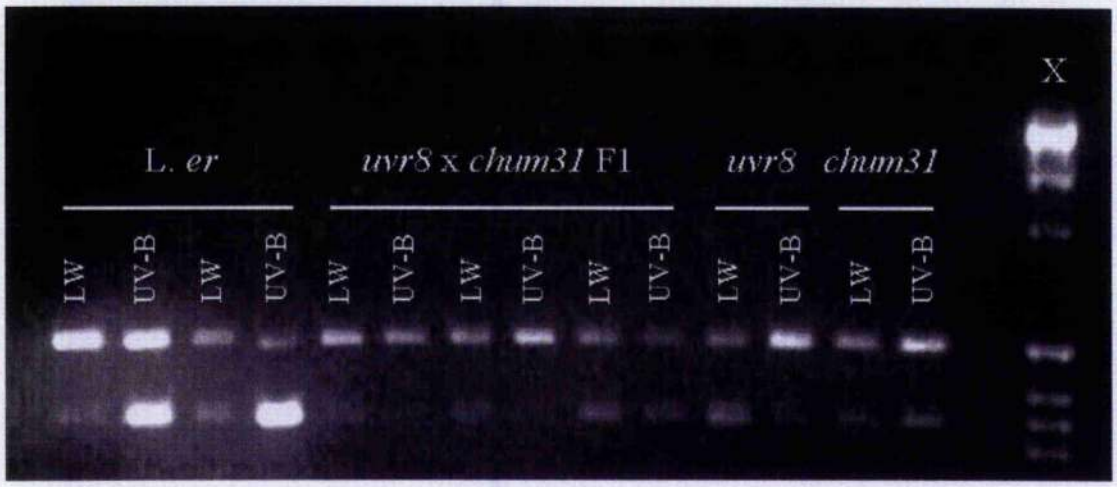


Figure 4.13
The *chum31* and *uvr8* mutants are deficient in allelic genes. Reverse transcriptase (RT)-PCR analysis of *CHS* mRNA induction by UV-B. F_1 plant tissue from the cross shown together with the appropriate parents was grown up under non-*CHS* inducing conditions (18-35 $\mu Em^{-2}s^{-1}$ white light) for 3 weeks before treatment with 3 $\mu Em^{-2}s^{-1}$ UV-B for 4 hours. Tissue was then harvested for RNA extraction and ethidium bromide stained products are shown from quantitative RT-PCR depicting *CHS* expression (lower bands) compared to an *ACTIN* loading control (upper bands). Each F_1 lane corresponds to *uvr8* x *chum31* F_1 tissue produced from a separate cross. The furthermost (left hand side) two L. *er* lanes (one lane each of LW and UV-B) feature identically treated tissue generated in a separate experiment from that represented in the other twelve lanes.

The cDNA sequences corresponding to the four mutant *uvr8* alleles uncovered by the screen have since been determined. The sizes of the transcripts are unaltered, however each allele appears to contain a 'G' to 'A' single nucleotide change. This mutation is the most frequent alteration produced by EMS mutagenesis in *Arabidopsis* (S. Naito, *Arabidopsis* Newsgroup Correspondence, 2002, http://arabi4.agr.hokudai.ac.jp/ArabiE/arabie.html). Each of the mutations should produce a change in one of the codons specifying the 440 amino acid UVR8 polypeptide sequence. In three of the mutants a tryptophan residue is replaced by a premature STOP codon. These nonsense mutations occur at Trp-400 in *chum31* (Cloix, Jiang and Jenkins, unpublished work), Trp-39 in *chum33* and Trp-302 in *chum53*. Interestingly, the weak *chum75* allele which still permits a little UV-B induced *CHS* expression does not contain a nonsense codon but a missense mutation. The incorporation of a large acidic glutamate residue replacing Gly-283 in the *chum75* protein appears to damage but not extirpate UVR8 function.

4.4.2 The *uvr8-1* and *chum31* Mutants Show a Similar Degree of Increased Susceptibility to Supplementary UV-B Induced Growth Inhibition and Leaf Damage

The *uvr8-1* mutant was originally isolated from a screen for mutants which showed an enhanced susceptibility to supplementary UV-B induced leaf damage (Kliebenstein et al., 2002). The possibility that the *uvr8-1* mutant is deficient in the perception or transduction of a UV-B light signal is raised by the observation that UV-B induced *CHS* expression is also reduced in *uvr8-1*. Perhaps an inability to detect or transduce the UV-B signal results in a reduction in the accumulation of protective pigments (such as flavonoids) which have the capacity to screen out harmful UV-B. Unsurprisingly, since the corresponding mutations are allelic, *chum31* shows a similar level of susceptibility to supplementary UV-B induced growth inhibition and leaf damage to that displayed by *uvr8-1* (Figure 4.14).

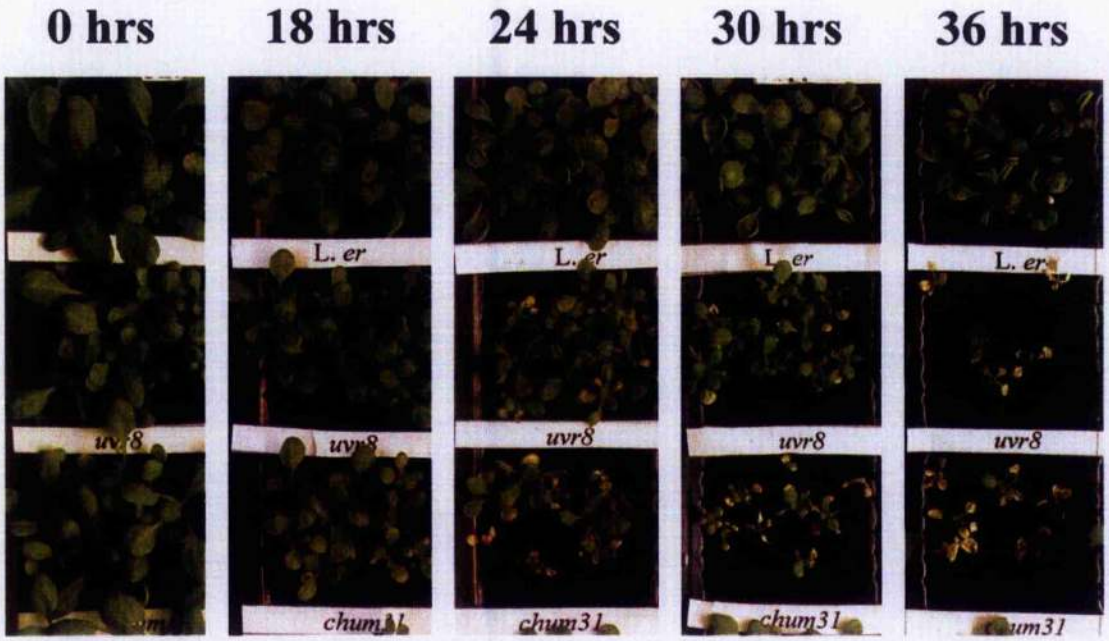


Figure 4.14
The *uvr8-1* and *chum31* mutants are equally susceptible to supplementary UV-B induced tissue damage. Wild-type L. *er* (top of each panel), *uvr8-1* (middle of each panel) and *chum31* (bottom of each panel) seedlings were grown up under 120 $\mu Em^{-2}s^{-1}$ white light for 12 days before treatments of varying durations (as indicated) with 5 $\mu Em^{-2}s^{-1}$ UV-B (Supplemented with 40 $\mu Em^{-2}s^{-1}$ white light). The pictures shown were taken after a 5 day recovery period in 120 $\mu Em^{-2}s^{-1}$ white light.

4.4.3 Two *chum31* Double Backcrossed Lines (*chum31bc2*) were Generated for Further Characterization of the Mutant

In order to generate publishable data using our novel mutant allele, double backcrossed lines were created from the *chum31* mutant. Backcrossing should remove additional, unwanted mutations and eliminate the *CHS-Luc* transgene. The *chum31* mutant was crossed to the *CHS-Luc3.4* (parent) line; several of the resulting F_1 plants were then crossed to wild-type L. *er* and F_2 seed generated from this latter series of crosses. Twenty-five of the resulting F_2 plants which did not express luciferase were retained and their progeny, in turn, tested to see which of the 25 lines exhibited the characteristic mutant low *CHS* expression phenotype in response to UV-B (Figure 4.15). As a result, two double backcrossed lines (*chum31bc2*) were generated and *chum31bc2* line 1 was used in many of the subsequent characterization experiments.

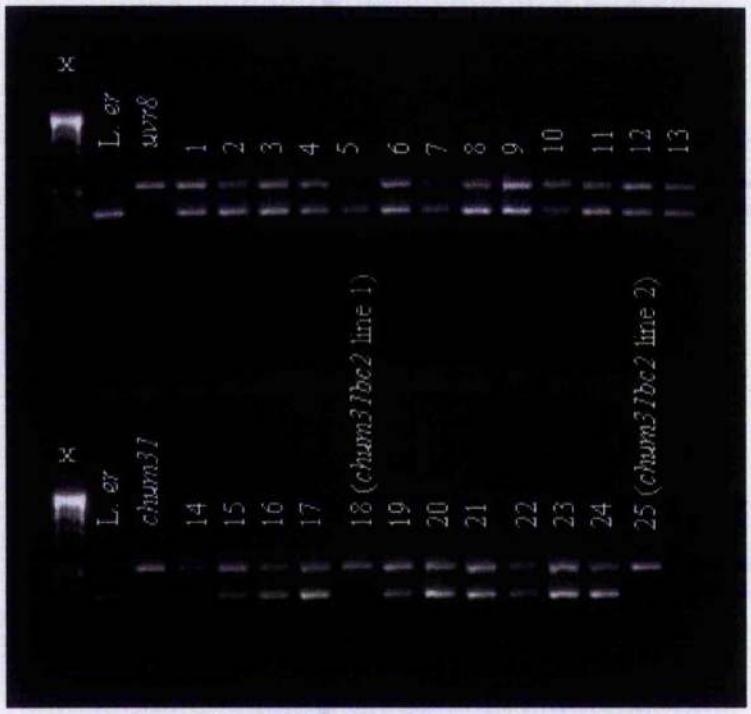


Figure 4.15
Two *chum31bc2* (double backcrossed) lines were identified for use in subsequent work. Reverse transcriptase (RT)-PCR analysis of *CHS* mRNA induction by UV-B. Putative double backcrossed mutant lines together with appropriate controls were grown up under non-*CHS* inducing conditions (18-35 $\mu Em^{-2}s^{-1}$ white light) for 3 weeks before treatment with 3 $\mu Em^{-2}s^{-1}$ UV-B for 4 hours. Tissue was then harvested for RNA extraction and ethidium bromide stained products are shown from quantitative RT-PCR depicting *CHS* expression (lower bands) compared to an *ACTIN* loading control (upper bands).

4.4.4 *CHS* Expression Studies on *uvr8bc5* and *chum31bc2* Mature *Arabidopsis* Leaf Tissue Demonstrates that the UV-A and Cold Induction Pathways Remain in these Mutants

In mature *Arabidopsis* leaf tissue, *CHS* expression is induced in response to stimuli such as UV-A, UV-B and low temperature (Jenkins et al., 2001, Wade et al., 2003). In the present work, initial experiments indicated that mutations in the *CHUM31 / UVR8* gene interfered significantly with UV-B but not UV-A induced *CHS* expression in the mature leaf. Confirmation that only UV-B induced *CHS* expression is lost in the *chum31 / uvr8* mutant was obtained during studies on the backcrossed lines (Figure 4.16). The *uvr8-1* mutant had been backcrossed five times (D. J. Kliebenstein, personal communication). Additionally, it was shown that mutation of the *CHUM31 / UVR8* gene appears to have little or no effect on the induction of *CHS* expression by low temperature.

Figure 4.16
Reverse transcriptase (RT)-PCR analysis of *CHS* mRNA induction by UV-A, UV-B and low temperature. L. *er* wild-type, *uvr8bc5* and *chum31bc2* were grown up under non-*CHS* inducing conditions (18-35 $\mu Em^{-2}s^{-1}$ low white light) for 3 weeks before treatment with either 100 $\mu Em^{-2}s^{-1}$ UV-A for 6 hours, 3 $\mu Em^{-2}s^{-1}$ UV-B for 4 hours, 10°C in low white light for 24 hours (Cold) or no further treatment (LW). Tissue was then harvested for RNA extraction and ethidium bromide stained products are shown from quantitative RT-PCR depicting *CHS* expression (lower bands) compared to an *ACTIN* loading control (upper bands). Note that a small reduction in UV-A induced *CHS* gene expression is observed in the *uvr8* mutants.

4.4.5 Far-Red Light Induced *CHS* Expression Studies on *uvr8bc5* and *chum31bc2* Dark Grown *Arabidopsis* Seedlings Suggest that the Phytochrome A Pathway Remains in these Mutants

In *Arabidopsis*, phytochrome can also mediate *CHS* expression, but only in young seedlings (Kaiser et al., 1995, Jenkins et al., 2001). By treating 4 day old, etiolated *Arabidopsis* seedlings with far-red light it was demonstrated that the phyA pathway for *CHS* induction also remains largely unaffected in the *uvr8* and *chum31* mutants (Figure 4.17). The data obtained from experiments using mature leaf and now also etiolated seedlings increasingly suggests that the UVR8 protein plays a role in inducing *CHS* gene expression only as a specific rejoinder to UV-B illumination.

It should also be noted that despite several independent experiments (only data for Figure 4.17 is shown) it is unclear whether the UV-B induction of *CHS* expression is completely lost in *chum31bc2* / *uvr8bc5* mutant seedlings, whereas in mature mutant leaf tissue the UV-B stimulated *CHS* response is unambiguously lost (Figure 4.16). Therefore, the possibility that UVR8 fulfils its role only during a particular plant developmental stage cannot be ruled out.

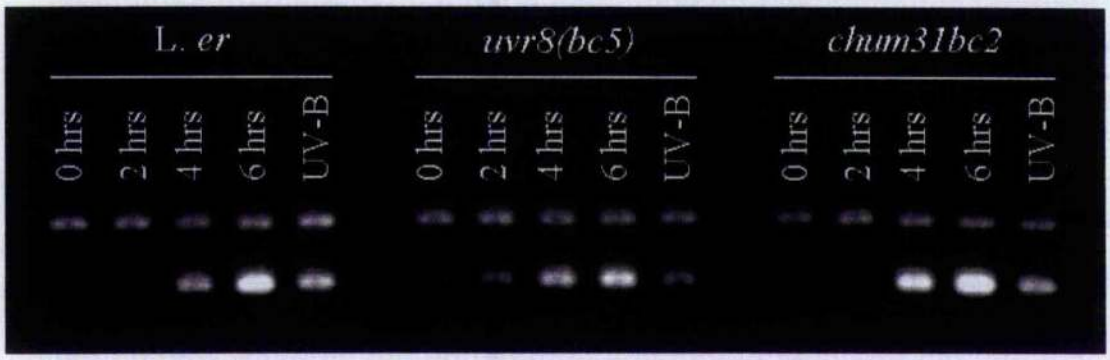


Figure 4.17
Reverse transcriptase (RT)-PCR analysis of *CHS* mRNA induction by UV-B and Far Red light. Four day old, dark grown L. *er* wild-type, *uvr8bc5* and *chum31bc2* seedlings (grown on media containing sucrose) were treated with 0, 2, 4 or 6 hours 70 $\mu Em^{-2}s^{-1}$ Far Red light or 4 hours of 3 $\mu Em^{-2}s^{-1}$ UV-B (as indicated). Tissue was then harvested for RNA extraction and ethidium bromide stained products are shown from quantitative RT-PCR depicting *CHS* expression (lower bands) compared to an *ACTIN* loading control (upper bands).

4.4.6 Genome-Wide Transcript Analysis Highlighted the Importance of the UVR8 Gene Product in the Response of Mature *Arabidopsis thaliana* Leaf Tissue to UV-B Treatment

4.4.6.1 Microarray Analysis was Performed Using the *Arabidopsis* ATH Array and FunAlyse Software to Identify Genes Regulated by UVR8 in Response to UV-B Treatment

RNA taken from three separate experiments was hybridized to *Arabidopsis* (ATH) Affymetrix chips (see Materials and Methods Section) and the data generated analysed by rank product statistics (Breitling et al., 2004) to identify genes which were differentially expressed in response to UV-B treatment in *uvr8* mutant and wild-type plants respectively. The microarray procedure and data analysis was performed at the University of Glasgow by staff of the Sir Henry Wellcome Functional Genomics Facility (see Materials and Methods Section). The interpretation of the data presented here was further based on advice from Dr. Pawel Herzyk (University of Glasgow). In each experiment 3 week old, low white light (18-35 $\mu Em^{-2}s^{-1}$) grown, wild-type L. *er* and *uvr8* (backcrossed alleles) tissue was treated (UV-B) or not treated (LW) with 3 $\mu Em^{-2}s^{-1}$ UV-B for 4 hours before harvest. Results were obtained in triplicate and used for gene expression level comparisons between the original *uvr8-1* allele (Kliebenstein et al., 2002) and wild-type L. *er*, whereas the novel (*chum31bc2*) allele was represented by RNA from a single experiment. The gene expression profiles produced by the two mutant alleles were similar. Table 4.18 records the comparisons which were made.

Gene List #	Treatment	Baseline	Direction
1	L. *er* UV-B + L. *er* UV-B + L. *er* UV-B	L. *er* LW + L. *er* LW + L. *er* LW	Positive
2	L. *er* UV-B + L. *er* UV-B + L. *er* UV-B	L. *er* LW + L. *er* LW + L. *er* LW	Negative
3	*uvr8* LW + *uvr8* LW + *uvr8* LW	L. *er* LW + L. *er* LW + L. *er* LW	Positive
4	*uvr8* LW + *uvr8* LW + *uvr8* LW	L. *er* LW + L. *er* LW + L. *er* LW	Negative
5	*uvr8* UV-B + *uvr8* UV-B + *uvr8* UV-B	L. *er* UV-B + L. *er* UV-B + L. *er* UV-B	Positive
6	*uvr8* UV-B + *uvr8* UV-B + *uvr8* UV-B	L. *er* UV-B + L. *er* UV-B + L. *er* UV-B	Negative
7	*uvr8* UV-B + *uvr8* UV-B + *uvr8* UV-B	*uvr8* LW + *uvr8* LW + *uvr8* LW	Positive
8	*uvr8* UV-B + *uvr8* UV-B + *uvr8* UV-B	*uvr8* LW + *uvr8* LW + *uvr8* LW	Negative
9	*chum31bc2* LW	L. *er* LW + L. *er* LW + L. *er* LW	Positive
10	*chum31bc2* LW	L. *er* LW + L. *er* LW + L. *er* LW	Negative
11	*chum31bc2* UV-B	L. *er* UV-B + L. *er* UV-B + L. *er* UV-B	Positive
12	*chum31bc2* UV-B	L. *er* UV-B + L. *er* UV-B + L. *er* UV-B	Negative
13	*chum31bc2* UV-B	*chum31bc2* LW	Positive
14	*chum31bc2* UV-B	*chum31bc2* LW	Negative

Table 4.18
Details of the analysed "between group" comparisons designed to identify genes which are differentially expressed in response to UV-B treatment in mutant (*uvr8* and *chum31bc2*) and wild-type (L. *er*) mature *Arabidopsis* plants. LW signifies no UV-B treatment. Most studies were done in triplicate, as shown. Direction refers to the direction of the differential expression of the treatment with respect to the baseline (Positive – means a list of genes with significantly higher expression in the treatment group than in the baseline group; Negative – means a list of genes with significantly lower expression in the treatment group than in the baseline group). The microarray procedure and data analysis was performed at the University of Glasgow by staff of the Sir Henry Wellcome Functional Genomics Facility. Highlighted results were used to generate Tables 4.19 and 4.20.

4.4.6.2 Examination of the Data Generated by Microarray Analysis Identified Genes Expressed in Response to UV-B – Some of Which Appear to be Induced *via* UVR8

The microarray analysis of UV-B induced genes generated a huge amount of data, much of which could not be included in the limited space available in the present volume. To make the information more manageable, differentially expressed gene lists were cut at a false discovery rate (FDR) of 5% - thereby limiting the number of false positive genes, expected to be present in each list, to only 5%. It should be noted that significant findings may be missed as a result of this pragmatic step; for example a reduction in the UV-B induced expression level of the *FAH1* gene in the *uvr8* mutant (found in Gene List #6 occurring at a false discovery rate of 18.71%) could contribute to the increased sensitivity of the *uvr8* mutant to supplementary UV-B induced leaf damage - although it is likely that the reduced expression of the flavonoid biosynthesis genes (see below) also leads to such increased sensitivity.

It was decided to focus on the expression of UV-B induced rather than UV-B downregulated genes. Partly responsible for this decision was the fact that Gene List #5 is

extremely short, containing a total of eleven genes and only one of these (At2g28630) also appears in Gene List #2. Perhaps therefore, growing *Arabidopsis* under low fluence rate white light does not provide the best baseline for identifying genes downregulated by UV-B treatment.

Since previous results have shown that the UVR8 gene product is required for UV-B induced *CHS* gene expression, I decided to examine solely those genes induced in response to UV-B in wild-type plants (Gene List #1) and try to identify which of *these* genes were either no longer expressed in the *uvr8* mutant (ie. overlapped with Gene List #6) or were still expressed in the mutant (ie. overlapped with Gene List #7). In this way, it should be possible to determine which UV-B induced gene expression pathways are regulated *via* UVR8 (Table 4.19) and which are controlled independently of UVR8 (Table 4.20). The results are significant.

Although a great many genes are induced normally by UV-B in the absence of UVR8 (Table 4.20), the most striking pattern to emerge from the data is the number of flavonoid biosynthesis genes found in Table 4.19. The genes flavanone 3-hydroxylase (*F3H*), chalcone synthase (*CHS*), flavonol synthase 1 (*FLS1*), chalcone isomerase (*CHI*) and dihydroflavonol 4-reductase (*DFR*) all depend upon UVR8 for their upregulation in response to UV-B. The gene 4-coumarate-CoA ligase 3 (*4CL3*), occurring as part of the general phenylpropanoid pathway, additionally seems to be UV-B induced via UVR8. Thus, it would seem that UVR8 has a key role to play in upregulating the expression of many genes of the flavonoid biosynthetic pathway under UV-B.

The two most strongly expressed UVR8 regulated UV-B genes (Table 4.19) are the early light-induced protein (*ELIP*) and putative *ELIP* genes respectively. Since no *ELIP*s are found in Table 4.20, it may be that UVR8 has a crucial role to play in controlling the specific upregulation of these genes in response to UV-B illumination. ELIPs bind chlorophyll, absorb light and accumulate transiently in plants subjected to high light stress; they appear to have a photoprotective function and consequently the loss of UVR8 may leave plants susceptible to photooxidative damage under UV-B (Hutin et al., 2003). Studies in *Pisum sativum* have

concurred in showing that, unusually for photosynthetically related genes, *ELIP* expression is upregulated after UV-B treatment (Savenstrand et al., 2004). Curiously, a previous study in *Arabidopsis thaliana* showed that *ELIP*s are amongst the first genes to be induced during photomorphogenesis but that neither cryptochrome nor phototropin photoreceptors are required for the blue light induction of the two studied *Arabidopsis ELIP*s. Perhaps significantly for work presented here in Section 4.5, the transcription factor HY5 was also implicated in the upregulation of *Arabidopsis ELIP1* but not *ELIP2* (Harari-Steinberg et al., 2001).

Unsurprisingly, a number of genes which might function in alleviating oxidative stress are found in both Tables 4.19 and 4.20. These include putative and / or confirmed glutathione peroxidases and glutathione S-transferases (Brosche and Strid, 2003, Rentel and Knight, 2004). The putative glutathione peroxidase genes appear to be specifically regulated by UVR8 (Table 4.19). Two putative FtsH proteases also appear uniquely on the UVR8 regulated gene list. Such ATP-dependent zinc metalloproteases have been implicated in the turnover of the D1 component of photosystem II, which can become damaged under UV-B or high intensity white light (Lindahl et al., 2000, Bergo et al., 2003). Thus it may be that UVR8 has an important role to play in maintaining photosynthetic activity in the face of UV-B induced stress. In addition, UVR8 is clearly a critical factor in the repair of UV-B generated DNA damage since the CPD photolyase gene *PHR1* appears only in Table 4.19. The data from the microarray provides a clear indication that UVR8 is very important in the plant's defence against a variety of UV-B induced forms of damage.

Table 4.19 is also distinctive in having no fewer than four copies of a class of gene not found at all in the much larger Table 4.20. The transducin / WD-40 repeat family protein genes, found here being upregulated by UV-B *via* UVR8, fulfil a variety of roles in eukaryotes including signal transduction, RNA splicing and export, protein degradation, cell cycle regulation, transcriptional regulation and apoptosis. Most WD repeat proteins are involved in protein-protein interactions and they often have a seven bladed propeller-like structure (Madrona and Wilson, 2004). X-ray crystallography has shown that human RCC1

(regulator of chromatin condensation), which is very similar to UVR8 (see Section 4.6), also has a seven bladed propeller-like structure but the sequence and structure of the repeats differ from those of the WD-40 domain proteins (Renault et al., 1998).

Transcription factors known to be important in light induced signal transduction pathways, such as HY5 and HYH (Osterlund et al., 2000, Holm et al., 2002), are also found in Table 4.19. Although the microarray data presented here should be confirmed using northern blots or RT-PCR, the fact that these transcription factors appear to be regulated by UVR8 is extremely significant. Coupled with the finding that only *UVR8* alleles were uncovered in the present screen for mutants showing a reduction in UV-B stimulated *CHS* transcript accumulation, this observation suggests that UVR8 may be part of a very short signal transduction pathway mediating UV-B induced gene expression and that it performs a key role in UV-B photoperception or signalling. An elusive photoreceptor may finally be within reach.

Table 4.19

UV-B Induced Genes Controlled by UVR8. Genes induced in response to UV-B in wild-type plants (Gene List #1) which were no longer expressed (or induced to a significantly lesser extent) in the *uvr8* mutant (Gene List #6). Ranked by extent of expression loss in the *uvr8* mutant (ie. according to Gene List #6). Seventy-nine genes are shown in total. Results for the highlighted genes are equivocal because these genes appear in both Table 4.19 and Table 4.20.

Microarray preparation and data analysis was carried out by staff of the Sir Henry Wellcome Functional Genomics Facility at The University of Glasgow according to the procedure described in the Materials and Methods Section. Genes are ranked according to the most significant (#1 at the top of each list) to the least significant (at the bottom of each list). The Probe-set ID is the Affymetrix probe-set identifier; RP score is a measure of differential expression calculated by the rank product method (Breitling et al., 2004). FDR is an estimate of False Discovery Rate (each list of genes was cut at an FDR of 5% indicating the expected percentage of false positives). The FCrma is a mean fold-change over all possible between-chip comparisons contributing to a given between group comparison. The FCnom corresponds to the mean nominal fold-change obtained from the rma fold-changes using an equation over all possible between chip comparisons contributing to a given between-group comparison.

0	#Probe-set ID	RPscore	FDR	FCrma	FCnom	"TAIR"	"MIPS"	Gene Symbol	Title
1	258321_at	1.36	[redacted]	-143.51	-3362.03	At3g22840	At3g22840	ELIP	chlorophyll A-B binding family protein / early light-induced protein (ELIP)
2	245306_at	2.03	[redacted]	-84.22	-1364.40	At4g14690	At4g14690	---	chlorophyll A-B binding family protein / early light-induced protein, putative
3	252123_at	3.26	[redacted]	-59.20	-778.13	At3g51240	At3g51240	F3H	naringenin 3-dioxygenase / flavanone 3-hydroxylase (F3H)
4	253496_at	4.74	[redacted]	-36.65	-298.83	At4g31870	At4g31870	---	glutathione peroxidase, putative
5	250207_at	5.61	[redacted]	-33.78	-311.85	At5g13930	At5g13930	CHS	chalcone synthase / naringenin-chalcone synthase
6	249063_at	6.21	[redacted]	-27.45	-218.10	At5g44110	At5g44110	---	ABC transporter family protein
7	250533_at	6.55	[redacted]	-27.70	-223.71	At5g08640	At5g08640	FLS1	flavonol synthase 1 (FLS1)
8	249769_at	9.75	[redacted]	-19.43	-122.37	At5g24120	At5g24120	sigE	RNA polymerase sigma subunit SigE (sigE) / sigma-like factor (SIG5)
9	251020_at	10.56	[redacted]	-18.14	-110.64	At5g02270	At5g02270	---	ABC transporter family protein
10	246966_at	12.34	[redacted]	-15.83	-91.02	At5g24850	At5g24850	---	cryptochrome dash (CRYD)
11	245560_at	13.20	[redacted]	-14.72	-77.47	At4g15480	At4g15480	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
12	247463_at	13.30	[redacted]	-15.09	-80.03	At5g62210	At5g62210	---	embryo-specific protein-related
14	251658_at	16.93	[redacted]	-13.04	-66.94	At3g57020	At3g57020	---	strictosidine synthase family protein
15	252010_at	21.50	[redacted]	-10.82	-45.31	At3g52740	At3g52740	---	expressed protein
16	249191_at	22.76	[redacted]	-9.59	-35.13	At5g42760	At5g42760	---	O-methyltransferase N-terminus domain-containing protein
17	251727_at	24.24	[redacted]	-9.66	-37.47	At3g56290	At3g56290	---	expressed protein
18	250420_at	25.02	[redacted]	-9.73	-38.66	At5g11260	At5g11260	---	bZIP protein HY5 (HY5)
19	253039_at	25.21	[redacted]	-10.10	-43.11	At4g37760	At4g37760	---	squalene monooxygenase, putative / squalene epoxidase, putative
20	251827_at	25.35	[redacted]	-10.00	-41.96	At3g55120	At3g55120	CHI	chalcone-flavanone isomerase / chalcone isomerase (CHI)
21	246468_at	29.70	[redacted]	-8.79	-33.42	At5g17050	At5g17050	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
22	249215_at	30.37	[redacted]	-8.56	-31.55	At5g42800	At5g42800	---	dihydroflavonol 4-reductase (dihydrokaempferol 4-reductase) (DFR)
23	250083_at	31.79	[redacted]	-8.34	-30.29	At5g17220	At5g17220	---	glutathione S-transferase, putative
24	248049_at	32.05	[redacted]	-8.67	-33.27	At5g56090	At5g56090	---	cytochrome oxidase assembly family protein

25	259537_at	32.71		-8.40	-31.82	At1g12370	At1g12370	—	type II CPD photolyase PHR1 (PHR1)
26	263122_at	38.16		-7.67	-26.76	At1g78510	At1g78510	GPPS	solanesyl diphosphate synthase (SPS)
28	253943_at	39.29		-6.63	-19.39	At4g27030	At4g27030	—	expressed protein
29	262705_at	40.50		-6.72	-20.70	At1g16260	At1g16260	—	protein kinase family protein
30	249798_at	40.86		-7.48	-26.68	At5g23730	At5g23730	—	transducin family protein / WD-40 repeat family protein
31	260955_at	41.16		-6.64	-19.49	At1g06000	At1g06000	—	UDP-glucoronosyl/UDP-glucosyl transferase family protein
32	262626_at	41.91		-7.35	-25.56	At1g06430	At1g06430	—	FtsH protease, putative
34	250794_at	42.55		-7.00	-22.61	At5g05270	At5g05270	—	chalcone-flavanone isomerase family protein
36	248347_at	46.08		-6.03	-16.18	At5g52250	At5g52250	—	transducin family protein / WD-40 repeat family protein
37	252661_at	46.43		-6.62	-20.30	At3g44450	At3g44450	—	expressed protein
38	261907_at	46.62		-6.62	-20.93	At1g65060	At1g65060	4CL3	4-coumarate--CoA ligase 3 / 4-coumaroyl-CoA synthase 3 (4CL3)
43	253879_s_at	50.82		-6.42	-20.08	At4g27570	At4g27570	—	glycosyltransferase family protein
46	265634_at	60.31		-6.12	-19.20	At2g25530	At2g25530	—	AFG1-like ATPase family protein
47	255594_at	61.54		-5.86	-17.52	At4g01660	At4g01660	—	ABC1 family protein
48	264752_at	63.16		-5.57	-15.43	At1g23010	At1g23010	—	multi-copper oxidase type I family protein
50	258349_at	68.49		-4.91	-12.37	At3g17610	At3g17610	—	bZIP transcription factor family protein / HY5-like protein (HYH)
55	260072_at	76.12		-5.18	-14.59	At1g73650	At1g73650	—	expressed protein
56	267138_s_at	79.62		-5.06	-13.60	At2g38210	At2g38210	—	ethylene-responsive protein, putative
57	247779_at	81.34		-4.70	-11.45	At5g58760	At5g58760	—	transducin family protein / WD-40 repeat family protein
59	264102_at	83.96		-4.86	-12.80	At1g79270	At1g79270	—	expressed protein
63	256548_at	88.57		-4.78	-12.86	At3g14770	At3g14770	—	nodulin MtN3 family protein
64	246272_at	88.59		-4.65	-11.84	At4g37150	At4g37150	—	esterase, putative
66	265091_s_at	90.94		-4.48	-10.95	At1g03495	At1g03495	—	transferase family protein
69	264906_at	97.30		-4.40	-10.71	At2g17270	At2g17270	—	mitochondrial substrate carrier family protein
74	258167_at	105.76	0.19	-4.10	-9.50	At3g21560	At3g21560	—	UDP-glucosyltransferase, putative
75	267066_at	105.83		-4.06	-8.90	At2g41040	At2g41040	—	methyltransferase-related
76	251091_at	106.53		-4.31	-10.60	At5g01410	At5g01410	—	stress-responsive protein, putative
77	257713_at	106.88		-4.27	-10.69	At3g27380	At3g27380	sdh2-1	succinate dehydrogenase, iron-sulphur subunit, mitochondrial (SDH2-1)
79	266832_at	108.77		-4.08	-9.30	At2g30040	At2g30040	—	protein kinase family protein
82	262526_at	117.17		-4.08	-9.60	At1g17050	At1g17050	—	geranyl diphosphate synthase, putative / GPPS, putative / dimethylallyltransferase, putative / prenyl transferase, putative
83	247641_at	120.92		-4.05	-9.55	At5g60540	At5g60540	—	SNO glutamine amidotransferase family protein
85	258188_at	126.60		-3.92	-9.03	At3g17800	At3g17800	—	expressed protein
95	245936_at	137.71		-3.74	-8.38	At5g19850	At5g19850	—	hydrolase, alpha/beta fold family protein
96	266578_at	137.76		-3.49	-6.88	At2g23910	At2g23910	—	cinnamoyl-CoA reductase-related
101	246954_at	145.26		-3.66	-8.23	At5g04830	At5g04830	—	expressed protein
104	256425_at	152.04		-3.60	-8.07	At1g33560	At1g33560	—	disease resistance protein (CC-NBS-LRR class), putative
111	259964_at	162.76		-3.42	-7.23	At1g53680	At1g53680	—	glutathione S-transferase, putative
118	252984_at	182.28		-3.13	-5.84	At4g37990	At4g37990	ELI3-2	mannitol dehydrogenase, putative (ELI3-2)
123	247060_at	189.24		-3.19	-6.64	At5g66760	At5g66760	—	succinate dehydrogenase (ubiquinone) flavoprotein subunit, mitochondrial / flavoprotein subunit of complex II
125	265886_at	194.52		-3.13	-6.05	At2g25620	At2g25620	—	protein phosphatase 2C, putative / PP2C, putative
136	259226_at	226.80		-2.91	-5.69	At3g07700	At3g07700	—	ABC1 family protein
139	266292_at	228.06	1.07	-2.73	-4.63	At2g29350	At2g29350	—	tropinone reductase, putative / tropine dehydrogenase, putative

144	255468_at	234.26		-2.91	-5.71	At4g03020	At4g03020	---	transducin family protein / WD-40 repeat family protein
145	261064_at	234.65		-2.88	-5.55	At1g07510	At1g07510	---	FtsH protease, putative
168	258757_at	282.65		-2.56	-4.31	At3g10910	At3g10910	---	zinc finger (C3HC4-type RING finger) family protein
171	261450_s_at	290.75	1.78	-1.73	-1.67	At1g21110	At1g21110	---	O-methyltransferase, putative
182	265197_at	304.30	1.90	-2.57	-4.44	At2g36750	At2g36750	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
191	245392_at	320.28		-2.44	-3.90	At4g15680	At4g15680	---	glutaredoxin family protein
195	266097_at	326.12	2.19	-2.46	-4.15	At2g37970	At2g37970	---	SOUL heme-binding family protein
203	248138_at	341.24		-2.42	-4.23	At5g54960	At5g54960	---	pyruvate decarboxylase, putative
218	260881_at	385.07	3.03	-2.30	-3.42	At1g21550	At1g21550	---	calcium-binding protein, putative
230	258094_at	423.56		-2.22	-3.66	At3g14690	At3g14690	---	cytochrome P450, putative
238	254890_at	438.29		-2.21	-3.62	At4g11600	At4g11600	---	glutathione peroxidase, putative
250	259076_at	455.31		-2.20	-3.44	At3g02140	At3g02140	---	expressed protein
258	249233_at	475.48		-2.13	-3.35	At5g42150	At5g42150	---	expressed protein
270	264148_at	491.53		-1.94	-2.51	At1g02220	At1g02220	---	no apical meristem (NAM) family protein

Table 4.20

UV-B Induced Genes NOT Dependant on UVR8. Genes induced in response to UV-B in wild-type plants (Gene List #1) which were still induced in the *uvr8* mutant (Gene List #7). Ranked by extent of induced expression in the *uvr8* mutant (ie. according to Gene List #7). Five hundred and ten genes are shown in total. Results for the highlighted genes are equivocal because these genes appear in both Table 4.19 and Table 4.20.

Microarray preparation and data analysis was carried out by staff of the Sir Henry Wellcome Functional Genomics Facility at The University of Glasgow according to the procedure described in the Materials and Methods Section. See Table 4.19 legend for further explanation of the data presented.

0	#Probe-set ID	RPscore	FDR	FCrma	FCnom	"TAIR"	"MIPS"	Gene Symbol	Title
1	260522_x_at	1.00	0.00	325.84	13708.53	At2g41730	At2g41730	---	expressed protein
2	262518_at	2.55	0.00	231.57	7962.71	At1g17170	At1g17170	---	glutathione S-transferase, putative
3	261021_at	4.86	0.00	154.04	4359.89	At1g26380	At1g26380	---	FAD-binding domain-containing protein
4	263231_at	5.41	0.00	140.10	3332.24	At1g05680	At1g05680	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
5	258277_at	6.39	0.00	133.87	3328.14	At3g26830	At3g26830	---	cytochrome P450 71B15, putative (CYP71B15)
6	252265_at	8.14	0.00	121.94	2768.19	At3g49620	At3g49620	DIN11	2-oxoacid-dependent oxidase, putative (DIN11)
7	260706_at	9.51	0.00	115.73	2756.81	At1g32350	At1g32350	---	alternative oxidase, putative
8	263403_at	9.59	0.00	101.58	1985.18	At2g04040	At2g04040	---	MATE efflux family protein
9	259479_at	9.82	0.00	116.92	2843.61	At1g19020	At1g19020	---	expressed protein
10	248434_at	10.92	0.00	103.72	2119.69	At5g51440	At5g51440	hsp23.5-M	23.5 kDa mitochondrial small heat shock protein (HSP23.5-M)
11	263402_at	12.48	0.00	87.76	1583.31	At2g04050	At2g04050	---	MATE efflux family protein
12	266270_at	13.86	0.00	97.14	2087.85	At2g29470	At2g29470	---	glutathione S-transferase, putative
13	253046_at	14.47	0.00	92.81	1849.58	At4g37370	At4g37370	---	cytochrome P450, putative
14	252908_at	17.07	0.00	89.44	1851.60	At4g39670	At4g39670	---	expressed protein
15	256376_s_at	17.28	0.00	84.99	1665.97	At1g66690	At1g66690	---	S-adenosyl-L-methionine:carboxyl methyltransferase family protein
16	265499_at	18.17	0.00	82.07	1525.57	At2g15480	At2g15480	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
17	247573_at	21.92	0.00	67.24	1006.11	At5g61160	At5g61160	---	transferase family protein
18	266995_at	23.23	0.00	67.51	1051.20	At2g34500	At2g34500	---	cytochrome P450 family protein
19	261763_at	25.70	0.00	62.50	912.74	At1g15520	At1g15520	---	ABC transporter family protein
20	249770_at	26.57	0.00	64.00	996.68	At5g24110	At5g24110	---	WRKY family transcription factor
21	266071_at	27.64	0.00	62.79	945.81	At2g18680	At2g18680	---	expressed protein
22	263210_at	28.22	0.00	63.92	983.46	At1g10585	At1g10585	---	expressed protein
23	257206_at	31.60	0.00	57.89	869.87	At3g16530	At3g16530	---	legume lectin family protein
24	252131_at	31.69	0.00	61.61	968.38	At3g50930	At3g50930	---	AAA-type ATPase family protein
25	257264_at	33.18	0.00	60.84	973.41	At3g22060	At3g22060	---	receptor protein kinase-related
26	265725_at	33.50	0.00	60.92	964.27	At2g32030	At2g32030	---	GCN5-related N-acetyltransferase (GNAT) family protein
27	261240_at	33.61	0.00	50.58	625.85	At1g32940	At1g32940	---	subtilase family protein
28	247949_at	34.09	0.00	62.14	1038.20	At5g57220	At5g57220	---	cytochrome P450, putative
29	247435_at	35.07	0.00	51.22	655.40	At5g62480	At5g62480	---	glutathione S-transferase, putative
30	265501_at	37.04	0.00	52.33	707.96	At2g15490	At2g15490	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
31	263182_at	37.60	0.00	65.09	1154.29	At1g05575	At1g05575	---	expressed protein
32	262229_at	38.31	0.00	52.39	740.44	At1g68620	At1g68620	---	expressed protein
33	252334_at	41.28	0.00	46.38	565.79	At3g48850	At3g48850	---	mitochondrial phosphate transporter, putative
34	263515_at	41.56	0.00	46.07	554.19	At2g21640	At2g21640	---	expressed protein

35	265200_s_at	43.19	0.00	48.36	633.07	At2g36790	At2g36790	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
36	261892_at	43.63	0.00	52.03	782.13	At1g80840	At1g80840	---	WRKY family transcription factor
37	260560_at	43.67	0.00	45.02	548.62	At2g43590	At2g43590	---	chitinase, putative
38	245082_at	44.68	0.00	61.13	1043.94	At2g23270	At2g23270	---	expressed protein
39	266752_at	44.94	0.00	47.00	614.80	At2g47000	At2g47000	---	multidrug resistant (MDR) ABC transporter, putative
40	245193_at	46.38	0.00	43.17	493.68	At1g67810	At1g67810	---	Fe-S metabolism associated domain-containing protein
41	249494_at	46.76	0.00	45.49	573.65	At5g39050	At5g39050	---	transferase family protein
42	261242_at	48.96	0.00	41.57	458.50	At1g32960	At1g32960	---	subtilase family protein
43	249481_at	49.02	0.00	42.03	471.68	At5g38900	At5g38900	---	DSBA oxidoreductase family protein
44	246099_at	50.29	0.02	42.85	514.37	At5g20230	At5g20230	---	plastocyanin-like domain-containing protein
45	263401_at	50.94	0.02	42.13	483.13	At2g04070	At2g04070	---	MATE efflux family protein
46	267567_at	54.00	0.02	44.98	597.73	At2g30770	At2g30770	---	cytochrome P450 71A13, putative (CYP71A13)
47	253796_at	55.45	0.02	44.04	569.56	At4g28460	At4g28460	---	hypothetical protein
48	251884_at	59.24	0.04	42.05	529.06	At3g54150	At3g54150	---	embryo-abundant protein-related
49	259033_at	60.80	0.04	36.71	371.88	At3g09410	At3g09410	---	pectinacetylesterase family protein
50	246858_at	63.99	0.04	37.45	413.43	At5g25930	At5g25930	---	leucine-rich repeat family protein / protein kinase family protein
51	266368_at	64.11	0.04	36.58	381.73	At2g41380	At2g41380	---	embryo-abundant protein-related
52	248381_at	64.92	0.04	35.34	364.78	At5g51830	At5g51830	---	pfkB-type carbohydrate kinase family protein
53	254408_at	66.42	0.06	38.90	472.73	At4g21390	At4g21390	---	S-locus lectin protein kinase family protein
54	266290_at	67.61	0.07	35.56	383.06	At2g29490	At2g29490	---	glutathione S-transferase, putative
55	251176_at	67.82	0.07	35.25	365.67	At3g63380	At3g63380	ACA12	calcium-transporting ATPase, plasma membrane-type, putative / Ca(2+)-ATPase, putative (ACA12)
56	249942_at	69.52	0.07	35.22	375.99	At5g22300	At5g22300	NIT4	nitrilase 4 (NIT4)
57	246584_at	69.74	0.07	37.40	437.50	At5g14730	At5g14730	---	expressed protein
58	260804_at	70.01	0.07	46.13	664.27	At1g78410	At1g78410	---	VQ motif-containing protein
59	258452_at	70.20	0.07	33.71	329.33	At3g22370	At3g22370	AOX1A	alternative oxidase 1a, mitochondrial (AOX1A)
60	251248_at	73.70	0.08	33.12	346.13	At3g62150	At3g62150	---	multidrug resistant (MDR) ABC transporter, putative
61	266267_at	76.96	0.10	32.31	308.59	At2g29460	At2g29460	---	glutathione S-transferase, putative
62	258947_at	77.46	0.10	42.04	590.94	At3g01830	At3g01830	---	calmodulin-related protein, putative
63	265668_at	77.58	0.10	32.60	314.74	At2g32020	At2g32020	---	GCN5-related N-acetyltransferase (GNAT) family protein
64	257840_at	80.32	0.09	34.08	374.94	At3g25250	At3g25250	---	protein kinase family protein
65	259272_at	80.42	0.09	32.02	342.20	At3g01290	At3g01290	---	band 7 family protein
66	261216_at	81.45	0.09	31.37	291.61	At1g33030	At1g33030	---	O-methyltransferase family 2 protein
67	256252_at	83.31	0.09	29.13	256.34	At3g11340	At3g11340	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
68	258100_at	84.29	0.09	30.49	291.36	At3g23550	At3g23550	---	MATE efflux family protein
69	253268_s_at	85.34	0.09	28.65	246.23	At4g34135	At4g34135	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
70	267300_at	85.74	0.09	28.64	246.24	At2g30140	At2g30140	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
71	263475_at	85.81	0.08	33.11	359.13	At2g31945	At2g31945	---	expressed protein
72	253414_at	85.83	0.08	30.08	290.97	At4g33050	At4g33050	---	calmodulin-binding family protein
73	261005_at	85.96	0.08	31.48	320.62	At1g26420	At1g26420	---	FAD-binding domain-containing protein
74	259947_at	86.07	0.08	28.27	242.60	At1g71530	At1g71530	---	protein kinase family protein
75	265670_s_at	86.29	0.08	33.62	384.33	At2g32210	At2g32210	---	expressed protein
76	259428_at	86.58	0.08	34.11	383.72	At1g01560	At1g01560	---	mitogen-activated protein kinase, putative / MAPK, putative (MPK11)
77	262517_at	87.13	0.08	29.75	277.15	At1g17180	At1g17180	---	glutathione S-transferase, putative
78	265674_at	87.40	0.08	34.07	404.59	At2g32190	At2g32190	---	expressed protein

79	256245_at	87.95	0.06	30.18	324.52	At3g12580	At3g12580	---	heat shock protein 70, putative / HSP70, putative
80	266010_at	88.37	0.07	43.75	614.66	At2g37430	At2g37430	---	zinc finger (C2H2 type) family protein (ZAT11)
81	246406_at	91.02	0.07	30.10	292.63	At1g57650	At1g57650	---	disease resistance protein (NBS-LRR class), putative
82	262119_s_at	91.74	0.07	28.10	259.09	At1g02930	At1g02930	---	glutathione S-transferase, putative
83	255259_at	91.93	0.07	27.81	236.58	At4g05020	At4g05020	---	NADH dehydrogenase-related
84	262118_at	92.35	0.07	25.60	209.14	At1g02850	At1g02850	---	glycosyl hydrolase family 1 protein
85	252346_at	93.67	0.08	31.06	321.80	At3g48650	At3g48650	---	---
86	245076_at	94.01	0.08	25.65	212.09	At2g23170	At2g23170	---	auxin-responsive GH3 family protein
87	258787_at	95.66	0.10	35.43	423.51	At3g11840	At3g11840	---	U-box domain-containing protein
88	262381_at	96.28	0.10	28.12	273.97	At1g72900	At1g72900	---	disease resistance protein (TIR-NBS class), putative
89	256012_at	96.91	0.10	28.69	279.69	At1g19250	At1g19250	---	flavin-containing monooxygenase family protein / FMO family protein
90	249377_at	97.36	0.11	27.53	255.44	At5g40690	At5g40690	---	expressed protein
91	262099_s_at	98.08	0.11	32.19	354.72	At1g59500	At1g59500	---	auxin-responsive GH3 family protein
92	259040_at	106.37	0.14	34.14	400.90	At3g09270	At3g09270	---	glutathione S-transferase, putative
93	245173_at	106.67	0.14	24.31	197.21	At2g47520	At2g47520	---	AP2 domain-containing transcription factor, putative
94	261934_at	106.84	0.14	25.22	214.21	At1g22400	At1g22400	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
95	261192_at	107.30	0.14	24.69	199.24	At1g32870	At1g32870	---	no apical meristem (NAM) family protein
96	266017_at	108.71	0.14	25.69	223.70	At2g18690	At2g18690	---	expressed protein
97	259297_at	109.06	0.13	24.16	192.82	At3g05360	At3g05360	---	disease resistance family protein / LRR family protein
98	245976_at	109.39	0.13	32.56	365.91	At5g13080	At5g13080	---	WRKY family transcription factor
99	259841_at	109.48	0.13	25.15	215.40	At1g52200	At1g52200	---	expressed protein
100	254318_at	109.49	0.13	24.30	195.06	At4g22530	At4g22530	---	embryo-abundant protein-related
101	248332_at	110.01	0.13	23.23	184.86	At5g52640	At5g52640	HSP81-1	heat shock protein 81-1 (HSP81-1) / heat shock protein 83 (HSP83)
102	249125_at	110.04	0.13	23.09	178.13	At5g43450	At5g43450	---	2-oxoglutarate-dependent dioxygenase, putative
103	257918_at	111.05	0.13	32.22	360.62	At3g23230	At3g23230	---	ethylene-responsive factor, putative
104	245854_at	114.08	0.12	23.15	177.55	At5g13490	At5g13490	---	ADP, ATP carrier protein 2, mitochondrial / ADP/ATP translocase 2 / adenine nucleotide translocator 2 (ANT2)
105	266536_at	115.08	0.12	24.90	217.89	At2g16900	At2g16900	---	expressed protein
106	253044_at	115.38	0.13	30.78	331.20	At4g37290	At4g37290	---	expressed protein
107	256756_at	115.46	0.13	23.89	194.98	At3g25610	At3g25610	---	haloacid dehalogenase-like hydrolase family protein
108	246831_at	117.80	0.13	22.45	173.27	At5g26340	At5g26340	---	hexose transporter, putative
109	264746_at	118.36	0.13	24.20	206.50	At1g62300	At1g62300	---	WRKY family transcription factor
110	259875_s_at	118.78	0.13	23.15	194.53	At1g76690	At1g76690	OPR2	12-oxophytodienoate reductase (OPR2)
111	256576_at	118.91	0.13	24.81	219.66	At3g28210	At3g28210	---	zinc finger (AN1-like) family protein
112	249983_at	119.06	0.12	22.25	170.03	At5g18470	At5g18470	---	curculin-like (mannose-binding) lectin family protein
113	247327_at	119.31	0.12	21.72	158.75	At5g64120	At5g64120	---	peroxidase, putative
114	264400_at	119.94	0.12	26.68	250.66	At1g61800	At1g61800	---	glucose-6-phosphate/phosphate translocator, putative
115	260405_at	122.31	0.15	27.55	273.26	At1g69930	At1g69930	---	glutathione S-transferase, putative
116	261394_at	123.54	0.15	25.32	226.12	At1g79680	At1g79680	---	wall-associated kinase, putative
117	263948_at	123.56	0.15	28.11	289.27	At2g35980	At2g35980	---	harpin-induced family protein (YLS9) / HIN1 family protein / harpin-responsive family protein
118	247655_at	124.50	0.16	22.60	180.13	At5g59820	At5g59820	---	zinc finger (C2H2 type) family protein (ZAT12)
119	254922_at	125.10	0.17	30.11	321.61	At4g11370	At4g11370	---	zinc finger (C3HC4-type RING finger) family protein
120	255543_at	125.46	0.17	21.06	152.52	At4g01870	At4g01870	---	tolB protein-related
121	262085_at	128.46	0.17	24.59	231.90	At1g56060	At1g56060	---	expressed protein

122	260556_at	129.54	0.17	19.93	144.00	At2g43620	At2g43620	---	chitinase, putative
123	252345_at	131.55	0.17	25.66	275.20	At3g48640	At3g48640	---	expressed protein
124	258975_at	133.58	0.19	23.92	207.62	At3g01970	At3g01970	---	WRKY family transcription factor
125	255406_at	135.10	0.22	20.58	152.38	At4g03450	At4g03450	---	ankyrin repeat family protein
126	258203_at	136.11	0.23	26.96	263.22	At3g13950	At3g13950	---	expressed protein
127	248607_at	136.64	0.23	21.24	167.03	At5g49480	At5g49480	---	sodium-inducible calcium-binding protein (ACP1) / sodium-responsive calcium-binding protein (ACP1)
128	266070_at	137.94	0.23	23.31	210.62	At2g18660	At2g18660	---	expansin family protein (EXPR3)
129	248551_at	138.69	0.22	20.12	151.81	At5g50200	At5g50200	---	expressed protein
130	264042_at	139.04	0.22	18.77	125.83	At2g03760	At2g03760	---	steroid sulfotransferase, putative
131	257536_at	141.11	0.24	28.11	292.12	At3g02800	At3g02800	---	tyrosine specific protein phosphatase family protein
132	255753_at	141.26	0.23	22.80	192.90	At1g18570	At1g18570	---	myb family transcription factor (MYB51)
133	266364_at	141.36	0.23	25.06	236.10	At2g41230	At2g41230	---	expressed protein
134	263935_at	141.55	0.23	22.61	190.03	At2g35930	At2g35930	---	U-box domain-containing protein
135	262935_at	141.65	0.24	18.68	127.79	At1g79410	At1g79410	---	transporter-related
136	251400_at	141.84	0.24	20.81	165.28	At3g60420	At3g60420	---	expressed protein
137	258815_at	142.82	0.23	17.87	114.64	At3g04000	At3g04000	---	short-chain dehydrogenase/reductase (SDR) family protein
138	252076_at	143.08	0.23	20.77	177.32	At3g51660	At3g51660	---	macrophage migration inhibitory factor family protein / MIF family protein
139	259937_s_at	144.27	0.23	17.92	116.69	At1g71330	At1g71330	---	ABC transporter family protein
140	245329_at	145.94	0.24	20.22	162.43	At4g14365	At4g14365	---	zinc finger (C3HC4-type RING finger) family protein / ankyrin repeat family protein
141	261648_at	145.95	0.23	24.42	232.88	At1g27730	At1g27730	---	zinc finger (C2H2 type) family protein (ZAT10) / salt-tolerance zinc finger protein (STZ)
142	267028_at	146.84	0.23	22.30	196.35	At2g38470	At2g38470	---	WRKY family transcription factor
143	254241_at	147.38	0.24	19.07	135.27	At4g23190	At4g23190	---	protein kinase family protein
144	245252_at	147.61	0.24	26.94	287.39	At4g17500	At4g17500	---	ethylene-responsive element-binding protein 1 (ERF1) / EREBP-2 protein
145	248794_at	147.69	0.23	21.99	184.69	At5g47220	At5g47220	---	ethylene-responsive element-binding factor 2 (ERF2)
146	254447_at	152.12	0.26	18.49	128.67	At4g20860	At4g20860	---	FAD-binding domain-containing protein
147	251895_at	152.21	0.26	18.80	135.89	At3g54420	At3g54420	---	class IV chitinase (CHIV)
148	264866_at	152.39	0.26	22.54	201.00	At1g24140	At1g24140	---	matrixin family protein
149	246293_at	152.59	0.26	24.73	261.72	At3g56710	At3g56710	---	sigA-binding protein
150	252993_at	152.66	0.25	17.17	111.03	At4g38540	At4g38540	MO2	monooxygenase, putative (MO2)
151	256933_at	152.73	0.25	18.77	133.91	At3g22600	At3g22600	---	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
152	265221_s_at	152.87	0.26	23.50	208.05	At2g02010	At2g02010	---	glutamate decarboxylase, putative
153	260239_at	153.48	0.25	22.08	183.95	At1g74360	At1g74360	---	leucine-rich repeat transmembrane protein kinase, putative
154	259507_at	155.02	0.25	17.18	110.63	At1g43910	At1g43910	---	AAA-type ATPase family protein
155	246777_at	158.08	0.26	21.93	189.62	At5g27420	At5g27420	---	zinc finger (C3HC4-type RING finger) family protein
156	260015_at	160.55	0.28	16.84	106.96	At1g67980	At1g67980	---	caffeoyl-CoA 3-O-methyltransferase, putative
157	245711_at	162.24	0.29	20.83	173.14	At5g04340	At5g04340	---	zinc finger (C2H2 type) family protein
158	250670_at	163.36	0.30	16.38	102.96	At5g06860	At5g06860	PGIP1	polygalacturonase inhibiting protein 1 (PGIP1)
159	259911_at	168.41	0.34	15.48	93.16	At1g72680	At1g72680	CAD	cinnamyl-alcohol dehydrogenase, putative
160	245324_at	169.74	0.36	14.72	82.89	At4g17260	At4g17260	---	L-lactate dehydrogenase, putative
161	248164_at	170.03	0.37	24.90	252.80	At5g54490	At5g54490	---	calcium-binding EF-hand protein, putative
162	260648_at	170.83	0.36	15.38	91.01	At1g08050	At1g08050	---	zinc finger (C3HC4-type RING finger) family protein
163	260243_at	171.33	0.36	18.57	134.47	At1g63720	At1g63720	---	expressed protein
164	248686_at	172.02	0.36	15.75	98.41	At5g48540	At5g48540	---	33 kDa secretory protein-related
165	256017_at	172.13	0.36	19.94	163.48	At1g19180	At1g19180	---	expressed protein

166	263184_at	173.98	0.39	16.07	104.01	At1g05560	At1g05560	---	UDP-glucose transferase (UGT75B2)
167	260327_at	179.37	0.44	14.32	79.95	At1g63840	At1g63840	---	zinc finger (C3HC4-type RING finger) family protein
168	257951_at	181.57	0.45	15.91	101.89	At3g21700	At3g21700	---	expressed protein
169	251790_at	183.28	0.47	15.07	90.17	At3g55470	At3g55470	---	C2 domain-containing protein
170	267083_at	184.25	0.46	14.09	79.29	At2g41100	At2g41100	---	touch-responsive protein / calmodulin-related protein 3, touch-induced (TCH3)
171	259979_at	185.79	0.46	16.36	108.20	At1g76600	At1g76600	---	expressed protein
172	258682_at	187.34	0.50	15.61	98.81	At3g08720	At3g08720	---	serine/threonine protein kinase (PK19)
173	266800_at	188.32	0.50	20.40	165.71	At2g22880	At2g22880	---	VQ motif-containing protein
174	263931_at	190.56	0.55	16.96	122.36	At2g36220	At2g36220	---	expressed protein
175	247177_at	192.97	0.56	14.70	87.22	At5g65300	At5g65300	---	expressed protein
176	247026_at	192.99	0.55	27.56	290.77	At5g67080	At5g67080	---	protein kinase family protein
177	256337_at	195.34	0.56	14.01	79.80	At1g72060	At1g72060	---	expressed protein
178	248327_at	195.92	0.59	15.84	103.89	At5g52750	At5g52750	---	heavy-metal-associated domain-containing protein
179	264832_at	196.20	0.59	13.36	72.33	At1g03660	At1g03660	---	expressed protein
180	250293_s_at	197.05	0.59	12.79	65.50	At5g13360	At5g13360	---	auxin-responsive GH3 family protein
181	265572_at	197.43	0.60	21.21	191.96	At2g28210	At2g28210	---	carbonic anhydrase family protein
182	259975_at	198.16	0.60	13.57	75.19	At1g76470	At1g76470	---	cinnamoyl-CoA reductase family
184	249719_at	199.36	0.60	16.40	112.40	At5g35735	At5g35735	---	auxin-responsive family protein
185	267246_at	199.67	0.60	15.76	107.21	At2g30250	At2g30250	---	WRKY family transcription factor
186	264467_at	201.08	0.62	14.31	85.11	At1g10140	At1g10140	---	expressed protein
187	263228_at	201.72	0.63	18.23	138.03	At1g30700	At1g30700	---	FAD-binding domain-containing protein
188	259445_at	210.22	0.70	15.19	96.58	At1g02400	At1g02400	---	gibberellin 2-oxidase, putative / GA2-oxidase, putative
189	257654_at	210.35	0.70	15.61	111.26	At3g13310	At3g13310	---	DNAJ heat shock N-terminal domain-containing protein
190	261450_s_at	210.44	0.69	24.87	241.48	At1g21110	At1g21110	---	O-methyltransferase, putative
191	260567_at	210.98	0.70	12.09	59.74	At2g43820	At2g43820	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
192	256178_s_at	211.24	0.69	12.19	60.62	At1g51780	At1g51780	---	IAA-amino acid hydrolase 5 / auxin conjugate hydrolase (ILL5)
193	266292_at	211.35	0.69	13.27	73.58	At2g29350	At2g29350	---	tropinone reductase, putative / tropine dehydrogenase, putative
194	250287_at	212.12	0.69	12.12	60.22	At5g13330	At5g13330	---	AP2 domain-containing transcription factor family protein
195	251282_at	212.30	0.69	12.18	61.61	At3g61630	At3g61630	---	AP2 domain-containing transcription factor, putative
196	261023_at	215.77	0.70	12.94	70.77	At1g12200	At1g12200	---	flavin-containing monooxygenase family protein / FMO family protein
197	254759_at	217.02	0.71	11.96	59.07	At4g13180	At4g13180	---	short-chain dehydrogenase/reductase (SDR) family protein
198	254592_at	217.12	0.71	12.67	67.00	At4g18880	At4g18880	---	heat shock transcription factor 21 (HSF21)
199	257950_at	217.52	0.72	13.18	73.38	At3g21780	At3g21780	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
200	264868_at	217.59	0.72	12.36	64.56	At1g24090	At1g24090	---	RNase H domain-containing protein
201	254818_at	218.23	0.72	19.01	174.27	At4g12470	At4g12470	---	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
202	250983_at	218.58	0.72	13.60	79.34	At5g02780	At5g02780	In2-1	In2-1 protein, putative
203	260040_at	219.20	0.72	19.27	154.52	At1g68765	At1g68765	---	expressed protein
204	267008_at	220.28	0.74	11.93	60.12	At2g39350	At2g39350	---	ABC transporter family protein
205	258002_at	220.70	0.74	11.96	59.46	At3g28930	At3g28930	AIG2	avrRpt2-induced AIG2 protein (AIG2)
206	253890_s_at	220.97	0.73	11.81	58.37	At5g54100	At5g54100	---	band 7 family protein
207	262882_at	221.77	0.73	12.39	65.32	At1g64900	At1g64900	---	cytochrome P450, putative
208	260225_at	222.96	0.73	13.21	76.13	At1g74590	At1g74590	---	glutathione S-transferase, putative
209	258201_at	223.06	0.73	13.50	78.71	At3g13910	At3g13910	---	expressed protein

210	245038_at	223.67	0.74	12.61	67.16	At2g26560	At2g26560	---	patatin, putative
211	247532_at	224.90	0.74	15.63	104.68	At5g61560	At5g61560	---	protein kinase family protein
212	246214_at	225.18	0.74	11.69	57.59	At4g36990	At4g36990	---	heat shock factor protein 4 (HSF4) / heat shock transcription factor 4 (HSTF4)
213	266884_at	228.45	0.77	11.86	60.17	At2g44790	At2g44790	---	uclacyanin II
214	255941_at	229.63	0.77	11.66	58.38	At1g20350	At1g20350	---	mitochondrial import inner membrane translocase subunit Tim17, putative
215	254926_at	232.40	0.78	15.54	110.95	At4g11280	At4g11280	ACS6	1-aminocyclopropane-1-carboxylate synthase 6 / ACC synthase 6 (ACS6)
216	248322_at	232.49	0.78	18.39	149.94	At5g52760	At5g52760	---	heavy-metal-associated domain-containing protein
217	257644_at	233.10	0.77	12.87	71.59	At3g25780	At3g25780	---	allene oxide cyclase, putative / early-responsive to dehydration protein, putative / ERD protein, putative
218	258805_at	233.42	0.78	11.54	56.58	At3g04010	At3g04010	---	glycosyl hydrolase family 17 protein
219	257623_at	236.40	0.81	11.78	60.46	At3g26210	At3g26210	---	cytochrome P450 71B23, putative (CYP71B23)
220	266835_at	237.46	0.81	11.00	51.64	At2g29990	At2g29990	---	pyridine nucleotide-disulphide oxidoreductase family protein
221	251769_at	237.52	0.81	14.66	94.59	At3g55950	At3g55950	---	protein kinase family protein
222	267623_at	240.28	0.83	13.25	77.89	At2g39650	At2g39650	---	expressed protein
223	250793_at	243.70	0.86	11.96	63.40	At5g05600	At5g05600	---	oxidoreductase, 2OG-Fe(II) oxygenase family protein
224	246870_at	244.00	0.86	16.10	133.98	At5g26030	At5g26030	---	ferrochelatase I
225	259439_at	244.06	0.86	11.14	54.30	At1g01480	At1g01480	ACS2	1-aminocyclopropane-1-carboxylate synthase 2 / ACC synthase 2 (ACS2) (ACC1)
226	246018_at	244.76	0.86	11.99	64.01	At5g10695	At5g10695	---	expressed protein
227	258786_at	245.25	0.86	12.32	68.44	At3g11820	At3g11820	SYP121	syntaxin 121 (SYP121) / syntaxin-related protein (SYR1)
228	250666_at	245.72	0.86	12.73	72.59	At5g07100	At5g07100	---	WRKY family transcription factor
229	252827_at	245.94	0.87	13.17	82.29	At4g39950	At4g39950	---	cytochrome P450 79B2, putative (CYP79B2)
230	255733_at	246.61	0.87	13.59	83.78	At1g25400	At1g25400	---	expressed protein
231	253824_at	249.41	0.90	10.70	50.52	At4g27940	At4g27940	---	mitochondrial substrate carrier family protein
232	265276_at	250.20	0.91	10.86	51.88	At2g28400	At2g28400	---	expressed protein
233	249928_at	252.15	0.91	17.43	131.42	At5g22250	At5g22250	---	CCR4-NOT transcription complex protein, putative
234	247071_at	252.39	0.91	12.48	69.46	At5g66640	At5g66640	---	LIM domain-containing protein-related
235	246744_at	252.70	0.91	10.82	51.04	At5g27760	At5g27760	---	hypoxia-responsive family protein
236	264663_at	253.87	0.92	10.55	49.45	At1g09970	At1g09970	---	leucine-rich repeat transmembrane protein kinase, putative
237	263221_at	254.60	0.93	12.20	67.72	At1g30620	At1g30620	---	UDP-D-xylose 4-epimerase, putative (MUR4)
238	256366_at	255.70	0.94	11.77	62.23	At1g66880	At1g66880	---	serine/threonine protein kinase family protein
239	261063_at	256.67	0.94	10.94	52.97	At1g07520	At1g07520	---	scarecrow transcription factor family protein
240	256647_at	256.88	0.94	10.89	52.63	At3g13610	At3g13610	---	oxidoreductase, 2OG-Fe(II) oxygenase family protein
241	254432_at	257.79	0.94	10.84	51.96	At4g20830	At4g20830	---	FAD-binding domain-containing protein
242	262911_s_at	257.88	0.94	11.46	61.01	At1g59860	At1g59860	hsp17.6A-CI	17.6 kDa class I heat shock protein (HSP17.6A-CI)
243	254549_at	258.34	0.94	10.31	47.09	At4g19880	At4g19880	---	glutathione S-transferase-related
244	246821_at	258.76	0.94	11.55	59.62	At5g26920	At5g26920	---	calmodulin-binding protein
245	247554_at	260.40	0.95	11.47	58.81	At5g61010	At5g61010	---	exocyst subunit EXO70 family protein
246	262213_at	261.00	0.94	14.91	99.99	At1g74870	At1g74870	---	expressed protein
247	264645_at	261.13	0.94	11.48	58.95	At1g08940	At1g08940	---	phosphoglycerate/bisphosphoglycerate mutase family protein
248	265061_at	263.15	0.95	10.48	48.46	At1g61640	At1g61640	---	ABC1 family protein
249	252470_at	265.65	0.96	11.22	57.39	At3g46930	At3g46930	---	protein kinase family protein
250	252921_at	266.83	0.96	11.47	59.42	At4g39030	At4g39030	EDS5/SID1	enhanced disease susceptibility 5 (EDS5) / salicylic acid induction deficient 1 (SID1)
251	266618_at	267.05	0.97	10.17	46.18	At2g35480	At2g35480	---	expressed protein

252	250575_at	267.15	0.97	10.96	54.44	At5g08240	At5g08240	---	expressed protein
253	246370_at	267.68	0.96	14.31	93.22	At1g51920	At1g51920	---	expressed protein
254	265615_at	268.27	0.96	11.84	70.98	At2g25450	At2g25450	---	2-oxoglutarate-dependent dioxygenase, putative
255	260568_at	270.56	0.96	11.84	65.53	At2g43570	At2g43570	---	chitinase, putative
256	254805_at	271.81	0.96	10.97	56.80	At4g12480	At4g12480	---	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
257	258606_at	272.55	0.99	18.67	149.48	At3g02840	At3g02840	---	immediate-early fungal elicitor family protein
258	250580_at	275.34	1.00	10.18	48.64	At5g07440	At5g07440	---	glutamate dehydrogenase 2 (GDH2)
259	265428_at	279.74	1.07	9.70	42.61	At2g20720	At2g20720	---	pentatricopeptide (PPR) repeat-containing protein
260	248330_at	280.81	1.07	10.58	52.21	At5g52810	At5g52810	---	ornithine cyclodeaminase/mu-crystallin family protein
261	256981_at	284.38	1.13	10.11	46.33	At3g13380	At3g13380	---	leucine-rich repeat family protein / protein kinase family protein
262	247351_at	284.52	1.13	12.70	81.05	At5g63790	At5g63790	---	no apical meristem (NAM) family protein
263	263096_at	286.02	1.13	10.81	55.96	At2g16060	At2g16060	AHB1/GLB1	non-symbiotic hemoglobin 1 (HB1) (GLB1)
264	247864_s_at	287.15	1.14	9.29	38.82	At5g57890	At5g57890	ASB	anthranilate synthase beta subunit, putative
265	247145_at	287.64	1.14	12.53	74.03	At5g65600	At5g65600	---	legume lectin family protein / protein kinase family protein
266	263073_at	288.74	1.15	9.31	39.54	At2g17500	At2g17500	---	auxin efflux carrier family protein
267	259705_at	290.91	1.16	9.83	44.86	At1g77450	At1g77450	---	no apical meristem (NAM) family protein
268	253332_at	291.13	1.16	9.29	39.24	At4g33420	At4g33420	---	peroxidase, putative
269	258507_at	291.29	1.16	9.07	37.40	At3g06500	At3g06500	---	beta-fructofuranosidase, putative / invertase, putative / saccharase, putative / beta-fructosidase, putative
270	254204_at	291.65	1.16	10.05	46.85	At4g24160	At4g24160	---	hydrolase, alpha/beta fold family protein
271	261027_at	291.67	1.16	9.70	43.07	At1g01340	At1g01340	---	cyclic nucleotide-regulated ion channel (CNGC10) (ACBK1)
272	260005_at	292.34	1.16	14.79	100.16	At1g67920	At1g67920	---	---
273	252053_at	294.40	1.16	10.47	50.87	At3g52400	At3g52400	SYP122	syntaxin, putative (SYP122)
274	245765_at	295.77	1.17	10.40	50.24	At1g33600	At1g33600	---	leucine-rich repeat family protein
275	257785_at	295.80	1.16	10.10	47.29	At3g26980	At3g26980	---	ubiquitin family protein
276	267288_at	296.35	1.17	10.29	49.64	At2g23680	At2g23680	---	stress-responsive protein, putative
277	253173_at	301.94	1.23	10.15	48.19	At4g35110	At4g35110	---	expressed protein
278	252300_at	302.41	1.23	11.70	67.32	At3g49160	At3g49160	---	pyruvate kinase family protein
279	250279_at	303.42	1.24	9.33	40.65	At5g13200	At5g13200	---	GRAM domain-containing protein / ABA-responsive protein-related
280	259403_at	305.28	1.25	9.12	40.71	At1g17745	At1g17745	---	D-3-phosphoglycerate dehydrogenase / 3-PGDH
281	254784_at	311.23	1.32	9.00	37.53	At4g12720	At4g12720	---	MutT/nudix family protein
282	265450_at	311.38	1.32	9.50	42.37	At2g46620	At2g46620	---	AAA-type ATPase family protein
283	246368_at	313.00	1.33	9.56	43.32	At1g51890	At1g51890	---	leucine-rich repeat protein kinase, putative
284	266761_at	314.06	1.33	9.57	43.37	At2g47130	At2g47130	---	short-chain dehydrogenase/reductase (SDR) family protein
285	250289_at	314.51	1.33	8.91	37.02	At5g13190	At5g13190	---	expressed protein
286	261526_at	316.42	1.33	10.05	48.56	At1g14370	At1g14370	---	protein kinase (APK2a)
287	261836_at	317.57	1.34	11.34	62.50	At1g16090	At1g16090	---	wall-associated kinase-related
288	261718_at	317.87	1.33	9.49	42.73	At1g18390	At1g18390	---	protein kinase family protein
289	253776_at	317.92	1.33	8.36	32.77	At4g28390	At4g28390	---	ADP, ATP carrier protein, mitochondrial, putative / ADP/ATP translocase, putative / adenine nucleotide translocator, putative
290	257216_at	318.17	1.34	8.43	33.08	At3g14990	At3g14990	---	4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein, putative
291	262072_at	318.38	1.34	9.63	44.39	At1g59590	At1g59590	---	expressed protein
292	250252_at	320.03	1.35	8.42	33.12	At5g13750	At5g13750	---	transporter-related
293	249987_at	320.07	1.35	9.91	47.15	At5g18490	At5g18490	---	expressed protein
294	258921_at	320.24	1.35	8.80	36.56	At3g10500	At3g10500	---	no apical meristem (NAM) family protein
295	255479_at	320.27	1.35	9.15	40.65	At4g02380	At4g02380	---	late embryogenesis abundant 3 family protein /

									LEA3 family protein
296	255595_at	324.14	1.36	9.56	43.94	At4g01700	At4g01700	---	chitinase, putative
297	248769_at	324.23	1.36	9.39	42.26	At5g47730	At5g47730	---	SEC14 cytosolic factor, putative / polyphosphoinositide-binding protein, putative
298	253203_at	324.34	1.36	10.23	54.39	At4g34710	At4g34710	SPE2	arginine decarboxylase 2 (SPE2)
299	249527_at	325.11	1.36	8.81	37.29	At5g38710	At5g38710	---	proline oxidase, putative / osmotic stress-responsive proline dehydrogenase, putative
300	264756_at	326.99	1.36	11.22	62.44	At1g61370	At1g61370	---	S-locus lectin protein kinase family protein
301	259626_at	327.08	1.38	9.69	45.26	At1g42990	At1g42990	---	bZIP transcription factor family protein
302	259211_at	328.80	1.38	10.02	49.54	At3g09020	At3g09020	---	alpha 1,4-glycosyltransferase family protein / glycosyltransferase sugar-binding DXD motif-containing protein
303	251975_at	329.90	1.39	9.10	41.78	At3g53230	At3g53230	---	cell division cycle protein 48, putative / CDC48, putative
304	264951_at	331.65	1.40	8.83	37.13	At1g76970	At1g76970	---	VHS domain-containing protein / GAT domain-containing protein
305	256306_at	333.15	1.41	10.24	53.25	At1g30370	At1g30370	---	lipase class 3 family protein
306	249417_at	334.79	1.42	9.77	46.93	At5g39670	At5g39670	---	---
307	265197_at	337.31	1.45	8.51	34.53	At2g36750	At2g36750	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
308	266615_s_at	338.21	1.45	8.36	33.28	At2g29720	At2g29720	---	monooxygenase family protein
309	247707_at	338.24	1.45	9.11	40.55	At5g59450	At5g59450	---	scarecrow-like transcription factor 11 (SCL11)
310	263807_at	339.44	1.46	7.90	29.74	At2g04400	At2g04400	IGPS	indole-3-glycerol phosphate synthase (IGPS)
311	252421_at	340.58	1.47	9.80	47.96	At3g47540	At3g47540	---	chitinase, putative
312	260387_at	340.78	1.47	8.07	31.46	At1g74100	At1g74100	---	sulfotransferase family protein
313	246247_at	342.59	1.48	8.41	34.41	At4g36640	At4g36640	---	SEC14 cytosolic factor family protein / phosphoglyceride transfer family protein
314	251910_at	343.07	1.47	8.84	38.24	At3g53810	At3g53810	---	lectin protein kinase, putative
315	245599_at	344.39	1.49	9.03	39.85	At4g14220	At4g14220	---	zinc finger (C3HC4-type RING finger) family protein
316	259520_at	344.68	1.49	8.97	39.63	At1g12320	At1g12320	---	expressed protein
317	257038_at	344.88	1.49	8.55	35.19	At3g19260	At3g19260	---	longevity-assurance (LAG1) family protein
318	245641_at	345.31	1.49	10.00	49.58	At1g25370	At1g25370	---	expressed protein
319	266167_at	345.76	1.50	8.10	31.48	At2g38860	At2g38860	---	proteasel (pfpI)-like protein (YLS5)
320	261748_at	347.14	1.52	10.72	56.64	At1g76070	At1g76070	---	expressed protein
321	255923_at	348.15	1.53	8.30	33.16	At1g22180	At1g22180	---	SEC14 cytosolic factor family protein / phosphoglyceride transfer family protein
322	257058_at	353.89	1.59	7.84	30.01	At3g15352	At3g15352	---	cytochrome c oxidase copper chaperone-related
323	256922_at	355.12	1.60	8.33	34.35	At3g19010	At3g19010	---	oxidoreductase, 2OG-Fe(II) oxygenase family protein
324	255430_at	356.00	1.62	7.69	29.47	At4g03320	At4g03320	---	chloroplast protein import component-related
325	266746_s_at	358.08	1.64	7.74	29.04	At2g02930	At2g02930	---	glutathione S-transferase, putative
326	267559_at	359.05	1.65	7.83	29.83	At2g45570	At2g45570	---	cytochrome P450 76C2, putative (CYP76C2) (YLS6)
327	257784_at	361.38	1.67	8.81	38.82	At3g26970	At3g26970	---	ubiquitin family protein
328	266658_at	362.95	1.68	12.24	75.07	At2g25735	At2g25735	---	expressed protein
329	245277_at	365.70	1.71	11.41	67.63	At4g15550	At4g15550	IAGLU	UDP-glucose:indole-3-acetate beta-D-glucosyltransferase (IAGLU)
330	267142_at	366.21	1.71	8.19	33.16	At2g38290	At2g38290	AMT2	ammonium transporter 2 (AMT2)
331	265723_at	368.27	1.75	12.26	73.68	At2g32140	At2g32140	---	disease resistance protein (TIR class), putative
332	259037_at	372.26	1.80	7.67	30.29	At3g09350	At3g09350	---	armadillo/beta-catenin repeat family protein
333	250781_at	373.42	1.80	7.98	32.48	At5g05410	At5g05410	---	DRE-binding protein (DREB2A)
334	254948_at	374.75	1.82	7.93	31.44	At4g11000	At4g11000	---	ankyrin repeat family protein
335	254321_at	375.37	1.82	7.26	26.12	At4g22590	At4g22590	---	trehalose-6-phosphate phosphatase, putative
336	258792_at	376.25	1.82	8.87	42.04	At3g04640	At3g04640	---	glycine-rich protein
338	252940_at	377.39	1.83	7.68	29.09	At4g39270	At4g39270	---	leucine-rich repeat transmembrane protein kinase, putative

339	248101_at	377.61	1.82	7.29	26.37	At5g55200	At5g55200	---	co-chaperone grpE protein, putative
340	251054_at	378.99	1.84	8.32	34.99	At5g01540	At5g01540	---	lectin protein kinase, putative
341	258915_at	379.30	1.84	7.88	31.18	At3g10640	At3g10640	---	SNF7 family protein
342	264757_at	380.68	1.87	8.05	32.56	At1g61360	At1g61360	---	S-locus lectin protein kinase family protein
343	262100_s_at	381.06	1.88	7.71	29.98	At1g59550	At1g59550	---	UBX domain-containing protein
344	247989_at	381.88	1.88	7.16	26.06	At5g56350	At5g56350	---	pyruvate kinase, putative
345	256522_at	382.13	1.88	7.57	28.93	At1g66160	At1g66160	---	U-box domain-containing protein
346	250267_at	384.27	1.88	7.24	25.96	At5g12930	At5g12930	---	expressed protein
347	263379_at	385.98	1.89	8.26	34.80	At2g40140	At2g40140	---	zinc finger (CCCH-type) family protein
348	263565_at	387.18	1.90	12.38	79.17	At2g15390	At2g15390	FUT4	xyloglucan fucosyltransferase, putative (FUT4)
349	266296_at	387.67	1.90	7.59	29.28	At2g29420	At2g29420	---	glutathione S-transferase, putative
350	249618_at	387.86	1.89	10.81	58.79	At5g37490	At5g37490	---	U-box domain-containing protein
351	248821_at	388.12	1.89	8.38	36.76	At5g47070	At5g47070	---	protein kinase, putative
352	266037_at	389.82	1.90	7.69	30.09	At2g05940	At2g05940	---	protein kinase, putative
353	265075_at	391.97	1.92	8.14	34.18	At1g55450	At1g55450	---	embryo-abundant protein-related
354	264460_at	392.62	1.92	7.27	26.43	At1g10170	At1g10170	---	NF-X1 type zinc finger family protein
355	246682_at	393.47	1.92	7.32	26.92	At5g33290	At5g33290	---	exostosin family protein
356	259213_at	395.82	1.95	10.55	56.15	At3g09010	At3g09010	---	protein kinase family protein
357	250738_at	396.08	1.94	6.98	24.29	At5g05730	At5g05730	ASA1	anthranilate synthase, alpha subunit, component I-1 (ASA1)
358	262571_at	398.73	1.97	7.94	32.27	At1g15430	At1g15430	---	expressed protein
359	266101_at	399.56	1.98	7.23	26.44	At2g37940	At2g37940	---	expressed protein
360	245393_at	400.14	1.98	7.04	24.80	At4g16260	At4g16260	---	glycosyl hydrolase family 17 protein
362	256442_at	401.30	1.99	8.35	36.66	At3g10930	At3g10930	---	expressed protein
363	257517_at	406.15	2.06	7.73	31.21	At3g16330	At3g16330	---	expressed protein
364	257621_at	407.09	2.06	7.11	25.60	At3g20410	At3g20410	---	calmodulin-domain protein kinase isoform 9 (CPK9)
365	245365_at	407.42	2.07	6.97	24.51	At4g17720	At4g17720	---	RNA recognition motif (RRM)-containing protein
366	267181_at	407.58	2.06	6.77	23.68	At2g37760	At2g37760	---	aldo/keto reductase family protein
367	264514_at	408.10	2.07	6.98	24.98	At1g09500	At1g09500	---	cinnamyl-alcohol dehydrogenase family / CAD family
368	257690_at	408.32	2.06	7.76	32.15	At3g12830	At3g12830	---	auxin-responsive family protein
369	264365_s_at	408.91	2.06	6.70	22.96	At1g03220	At1g03220	---	extracellular dermal glycoprotein, putative / EDGP, putative
371	258179_at	412.67	2.09	7.52	29.52	At3g21690	At3g21690	---	MATE efflux family protein
373	261443_at	412.79	2.08	9.49	46.91	At1g28480	At1g28480	---	glutaredoxin family protein
374	258880_at	413.40	2.09	7.12	26.09	At3g06420	At3g06420	APG8h	autophagy 8h (APG8h)
375	262219_at	414.56	2.11	7.61	30.26	At1g74750	At1g74750	---	pentatricopeptide (PPR) repeat-containing protein
376	264580_at	418.50	2.14	7.04	25.91	At1g05340	At1g05340	---	expressed protein
377	257061_at	418.75	2.13	9.62	48.17	At3g18250	At3g18250	---	expressed protein
382	256050_at	426.57	2.21	8.24	35.86	At1g07000	At1g07000	---	exocyst subunit EXO70 family protein
384	250094_at	427.66	2.21	6.42	21.51	At5g17380	At5g17380	---	pyruvate decarboxylase family protein
386	262607_at	434.95	2.32	6.34	21.04	At1g13990	At1g13990	---	expressed protein
387	266606_at	436.61	2.33	7.01	25.62	At2g46310	At2g46310	---	AP2 domain-containing transcription factor, putative
388	265199_s_at	437.06	2.34	7.10	26.53	At2g36770	At2g36770	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
389	260881_at	438.43	2.36	7.16	27.24	At1g21550	At1g21550	---	calcium-binding protein, putative
390	245662_at	440.11	2.36	9.61	49.67	At1g28190	At1g28190	---	expressed protein
391	246485_at	440.19	2.37	7.43	29.41	At5g16080	At5g16080	---	expressed protein
392	256633_at	440.27	2.37	10.07	53.89	At3g28340	At3g28340	---	galactinol synthase, putative
393	253628_at	441.04	2.37	11.70	78.70	At4g30280	At4g30280	---	xyloglucan:xyloglucosyl transferase, putative / xyloglucan endotransglucosylase, putative /

									endo-xyloglucan transferase, putative
394	246463_at	441.23	2.37	6.52	22.82	At5g16970	At5g16970	---	NADP-dependent oxidoreductase, putative (P1)
396	254707_at	442.85	2.38	6.28	20.36	At4g18010	At4g18010	IP5PII	inositol polyphosphate 5-phosphatase II (IP5PII)
397	248335_at	443.17	2.38	6.54	22.91	At5g52450	At5g52450	---	MATE efflux protein-related
398	260419_at	444.26	2.39	6.24	20.26	At1g69730	At1g69730	---	protein kinase family protein
400	261973_at	447.15	2.41	7.56	30.49	At1g64610	At1g64610	---	WD-40 repeat family protein
402	256453_at	447.72	2.41	6.40	21.28	At1g75270	At1g75270	---	dehydroascorbate reductase, putative
403	250796_at	449.45	2.44	9.71	49.45	At5g05300	At5g05300	---	expressed protein
404	267289_at	449.91	2.45	7.47	29.85	At2g23770	At2g23770	---	protein kinase family protein / peptidoglycan-binding LysM domain-containing protein
405	259925_at	450.68	2.45	9.54	52.92	At1g75040	At1g75040	PR-5	pathogenesis-related protein 5 (PR-5)
407	253779_at	451.28	2.44	6.42	21.82	At4g28490	At4g28490	---	leucine-rich repeat transmembrane protein kinase, putative
409	258063_at	452.72	2.45	6.23	20.86	At3g14620	At3g14620	---	cytochrome P450, putative
410	247835_at	454.34	2.47	6.85	25.17	At5g57910	At5g57910	---	expressed protein
411	258679_at	454.47	2.46	6.19	20.75	At3g08590	At3g08590	---	2,3-biphosphoglycerate-independent phosphoglycerate mutase, putative / phosphoglyceromutase, putative
412	253284_at	455.56	2.48	7.37	29.17	At4g34150	At4g34150	---	C2 domain-containing protein
413	254262_at	456.26	2.48	6.56	23.54	At4g23480	At4g23480	---	hydroxyproline-rich glycoprotein family protein
414	260415_at	456.75	2.49	6.22	20.60	At1g69790	At1g69790	---	protein kinase, putative
415	260477_at	458.62	2.50	7.47	30.20	At1g11050	At1g11050	---	protein kinase family protein
417	259980_at	460.97	2.53	6.15	20.01	At1g76520	At1g76520	---	auxin efflux carrier family protein
418	254077_at	461.12	2.52	6.41	22.09	At4g25640	At4g25640	---	MATE efflux family protein
419	264107_s_at	462.54	2.55	6.45	22.21	At2g13790	At2g13790	---	leucine-rich repeat family protein / protein kinase family protein
420	265479_at	464.99	2.58	7.16	27.73	At2g15760	At2g15760	---	calmodulin-binding protein
421	254741_s_at	465.57	2.59	8.25	38.93	At4g13900	At4g13900	---	disease resistance family protein / LRR family protein
422	253950_at	466.27	2.59	5.96	18.75	At4g26910	At4g26910	---	2-oxoacid dehydrogenase family protein
423	266181_at	470.67	2.66	5.94	19.11	At2g02390	At2g02390	---	glutathione S-transferase zeta 1 (GSTZ1) (GST18)
424	246098_at	471.27	2.66	7.04	27.53	At5g20400	At5g20400	---	oxidoreductase, 2OG-Fe(II) oxygenase family protein
425	255884_at	473.75	2.70	6.95	27.01	At1g20310	At1g20310	---	expressed protein
426	245686_at	473.79	2.70	6.09	19.73	At5g22060	At5g22060	---	DNAJ heat shock protein, putative
427	255230_at	474.04	2.69	5.94	18.79	At4g05390	At4g05390	---	ferredoxin--NADP(+) reductase, putative / adrenodoxin reductase, putative
428	254289_at	474.84	2.70	7.83	33.27	At4g22980	At4g22980	---	expressed protein
429	264867_at	474.99	2.69	8.16	37.37	At1g24150	At1g24150	---	formin homology 2 domain-containing protein / FH2 domain-containing protein
430	247930_at	477.69	2.73	6.37	21.95	At5g57060	At5g57060	---	expressed protein
431	248162_at	477.82	2.73	5.86	18.18	At5g54500	At5g54500	---	quinone reductase, putative
432	260418_s_at	480.06	2.75	6.11	19.99	At1g69750	At1g69750	---	cox19 family protein
433	260399_at	480.80	2.77	8.66	41.16	At1g72520	At1g72520	LOX	lipoxygenase, putative
435	255011_at	482.94	2.80	6.03	19.50	At4g10040	At4g10040	---	cytochrome c, putative
436	253013_at	485.07	2.81	5.79	18.16	At4g37910	At4g37910	---	heat shock protein 70, mitochondrial, putative / HSP70, mitochondrial, putative
437	261618_at	485.46	2.80	5.84	18.20	At1g33110	At1g33110	---	MATE efflux family protein
438	257902_at	485.50	2.79	5.93	18.80	At3g28450	At3g28450	---	leucine-rich repeat transmembrane protein kinase, putative
439	257830_at	486.15	2.80	5.71	17.56	At3g26690	At3g26690	---	MutT/nudix family protein
440	255502_at	487.07	2.81	6.73	24.91	At4g02410	At4g02410	---	lectin protein kinase family protein
441	266097_at	487.26	2.81	6.36	22.12	At2g37970	At2g37970	---	SOUL heme-binding family protein
442	252533_at	488.18	2.83	7.30	29.29	At3g46110	At3g46110	---	expressed protein
443	249485_at	491.29	2.89	7.62	31.75	At5g39020	At5g39020	---	protein kinase family protein

444	259879_at	491.68	2.89	8.35	37.94	At1g76650	At1g76650	---	calcium-binding EF hand family protein
445	246988_at	491.80	2.89	6.87	26.01	At5g67340	At5g67340	---	armadillo/beta-catenin repeat family protein / U-box domain-containing protein
446	252114_at	493.30	2.90	6.26	21.67	At3g51450	At3g51450	---	strictosidine synthase family protein
448	251479_at	494.21	2.90	8.36	38.37	At3g59700	At3g59700	---	lectin protein kinase, putative
449	267178_at	496.97	2.94	5.82	18.28	At2g37750	At2g37750	---	expressed protein
450	245119_at	497.23	2.94	8.56	39.78	At2g41640	At2g41640	---	expressed protein
451	253343_at	497.84	2.94	5.73	17.93	At4g33540	At4g33540	---	metallo-beta-lactamase family protein
454	265679_at	501.23	2.96	5.79	18.08	At2g32240	At2g32240	---	expressed protein
456	265999_at	502.83	2.98	5.91	19.36	At2g24100	At2g24100	---	expressed protein
458	249237_at	504.35	2.99	5.82	18.53	At5g42050	At5g42050	---	expressed protein
459	248060_at	506.61	3.02	5.84	18.59	At5g55560	At5g55560	---	protein kinase family protein
461	257700_at	508.05	3.03	5.95	19.36	At3g12740	At3g12740	---	LEM3 (ligand-effect modulator 3) family protein / CDC50 family protein
462	256793_at	508.32	3.02	6.65	24.57	At3g22160	At3g22160	---	VQ motif-containing protein
463	249252_at	508.54	3.02	6.46	23.18	At5g42010	At5g42010	---	WD-40 repeat family protein
464	247509_at	509.80	3.03	5.99	19.68	At5g62020	At5g62020	---	heat shock factor protein, putative (HSF6) / heat shock transcription factor, putative (HTSF6)
465	267624_at	510.54	3.03	6.43	23.21	At2g39660	At2g39660	---	protein kinase, putative
467	250994_at	512.59	3.07	5.72	18.04	At5g02490	At5g02490	---	heat shock cognate 70 kDa protein 2 (HSC70-2) (HSP70-2)
470	255039_at	516.91	3.12	5.71	17.90	At4g09570	At4g09570	---	calcium-dependent protein kinase, putative / CDPK, putative
471	251684_at	517.72	3.12	5.99	19.87	At3g56410	At3g56410	---	expressed protein
474	258282_at	525.38	3.24	6.69	25.06	At3g26910	At3g26910	---	hydroxyproline-rich glycoprotein family protein
476	245041_at	525.84	3.23	8.60	41.62	At2g26530	At2g26530	---	expressed protein
477	246305_at	528.03	3.25	5.53	16.93	At3g51890	At3g51890	---	expressed protein
478	245528_at	528.94	3.26	5.54	17.29	At4g15530	At4g15530	---	pyruvate phosphate dikinase family protein
479	254571_at	532.50	3.31	7.31	29.92	At4g19370	At4g19370	---	hypothetical protein
481	260602_at	536.26	3.34	6.95	27.35	At1g55920	At1g55920	---	serine O-acetyltransferase, putative
484	256183_at	537.61	3.34	6.44	23.40	At1g51660	At1g51660	---	mitogen-activated protein kinase kinase (MAPKK), putative (MKK4)
486	245247_at	539.40	3.36	5.37	15.93	At4g17230	At4g17230	---	scarecrow-like transcription factor 13 (SCL13)
487	265203_at	541.82	3.39	5.26	15.51	At2g36630	At2g36630	---	expressed protein
488	262360_at	542.01	3.38	5.87	19.59	At1g73080	At1g73080	---	leucine-rich repeat transmembrane protein kinase, putative
490	250818_at	542.61	3.38	5.42	16.30	At5g04930	At5g04930	ALA1	phospholipid-transporting ATPase 1 / aminophospholipid flippase 1 / magnesium-ATPase 1 (ALA1)
491	258979_at	546.21	3.43	5.48	17.25	At3g09440	At3g09440	---	heat shock cognate 70 kDa protein 3 (HSC70-3) (HSP70-3)
495	251259_at	553.17	3.50	5.48	17.18	At3g62260	At3g62260	---	protein phosphatase 2C, putative / PP2C, putative
498	251745_at	556.43	3.52	7.68	35.02	At3g55980	At3g55980	---	zinc finger (CCCH-type) family protein
499	249197_at	556.58	3.52	10.26	56.29	At5g42380	At5g42380	---	calmodulin-related protein, putative
500	254487_at	556.99	3.51	7.74	34.38	At4g20780	At4g20780	---	calcium-binding protein, putative
501	245051_at	560.51	3.55	5.44	16.69	At2g23320	At2g23320	---	WRKY family transcription factor
503	255895_at	565.31	3.64	5.11	14.62	At1g17990	At1g17990	OPR	12-oxophytodienoate reductase, putative
505	249001_at	569.27	3.70	7.12	29.52	At5g44990	At5g44990	---	hypothetical protein
507	264624_at	569.99	3.69	5.31	16.36	At1g08930	At1g08930	---	early-responsive to dehydration stress protein (ERD6) / sugar transporter family protein
509	248934_at	572.66	3.72	7.65	33.67	At5g46080	At5g46080	---	protein kinase family protein
510	250916_at	572.72	3.72	4.99	13.99	At5g03630	At5g03630	---	monodehydroascorbate reductase, putative
511	248207_at	573.25	3.72	5.06	14.62	At5g53970	At5g53970	---	aminotransferase, putative
513	257828_at	576.24	3.75	5.99	20.67	At3g26670	At3g26670	---	expressed protein
516	256430_at	578.25	3.90	5.03	14.30	At3g11020	At3g11020	---	DRE-binding protein (DREB2B)

517	245879_at	578.69	3.80	5.16	15.33	At5g09420	At5g09420	---	chloroplast outer membrane translocon subunit, putative
522	266000_at	591.53	3.98	4.98	14.06	At2g24180	At2g24180	---	cytochrome P450 family protein
525	267357_at	595.11	4.05	6.21	22.67	At2g40000	At2g40000	---	expressed protein
527	252278_at	597.15	4.08	6.38	25.00	At3g49530	At3g49530	---	no apical meristem (NAM) family protein
529	248550_at	599.23	4.12	4.84	13.53	At5g50210	At5g50210	---	quinolinate synthetase A-related
530	259749_at	599.81	4.12	6.58	25.43	At1g71100	At1g71100	---	ribose 5-phosphate isomerase-related
534	245613_at	600.50	4.10	7.56	33.08	At4g14450	At4g14450	---	expressed protein
535	261719_at	602.43	4.13	6.85	27.13	At1g18380	At1g18380	---	expressed protein
537	250934_at	602.55	4.11	4.78	13.28	At5g03030	At5g03030	---	DNAJ heat shock N-terminal domain-containing protein
541	245866_s_at	605.60	4.15	5.59	18.22	At1g57990	At1g57990	---	purine permease-related
542	254103_at	606.84	4.16	5.21	15.79	At4g25030	At4g25030	---	expressed protein
544	252102_at	607.88	4.16	5.39	17.72	At3g50970	At3g50970	---	dehydrin xero2 (XERO2) / low-temperature-induced protein LTI30 (LTI30)
553	247488_at	622.99	4.36	4.62	12.51	At5g61820	At5g61820	---	expressed protein
554	245755_at	623.67	4.36	10.78	61.19	At1g35210	At1g35210	---	expressed protein
555	249652_at	625.07	4.39	4.88	13.88	At5g37070	At5g37070	---	expressed protein
556	255677_at	628.86	4.47	4.85	13.86	At4g00500	At4g00500	---	lipase class 3 family protein / calmodulin-binding heat-shock protein-related
560	259473_at	633.91	4.52	5.74	19.82	At1g19025	At1g19025	---	DNA cross-link repair protein-related
562	251640_at	637.54	4.57	6.05	21.74	At3g57450	At3g57450	---	expressed protein
563	248487_at	638.00	4.57	4.62	12.46	At5g51070	At5g51070	---	ATP-dependent Clp protease ATP-binding subunit (ClpD), (ERD1)
566	249918_at	639.04	4.57	4.82	13.69	At5g19240	At5g19240	---	expressed protein
567	245249_at	639.43	4.58	4.52	12.03	At4g16760	At4g16760	ACX1	acyl-CoA oxidase (ACX1)
573	252487_at	643.21	4.58	5.05	15.68	At3g46660	At3g46660	---	UDP-glucoronosyl/UDP-glucosyl transferase family protein
579	258167_at	647.16	4.59	4.89	14.24	At3g21560	At3g21560	---	UDP-glucosyltransferase, putative
583	261445_at	649.81	4.62	5.18	16.00	At1g28380	At1g28380	---	expressed protein
585	256589_at	650.27	4.62	4.80	13.88	At3g28740	At3g28740	---	cytochrome P450 family protein
587	248713_at	652.73	4.65	4.53	12.21	At5g48180	At5g48180	---	kelch repeat-containing protein
589	251200_at	656.14	4.70	4.95	14.75	At3g63010	At3g63010	---	expressed protein
590	263419_at	656.57	4.71	4.51	12.05	At2g17220	At2g17220	---	protein kinase, putative
591	265359_at	657.89	4.72	4.55	12.33	At2g16720	At2g16720	---	myb family transcription factor
594	246270_at	659.30	4.72	5.81	20.03	At4g36500	At4g36500	---	expressed protein
595	253637_at	659.83	4.72	4.59	12.52	At4g30390	At4g30390	---	expressed protein
596	259992_at	660.53	4.72	5.15	16.15	At1g67970	At1g67970	---	heat shock factor protein, putative (HSF5) / heat shock transcription factor, putative (HSTF5)
598	252134_at	661.73	4.73	4.51	12.29	At3g50910	At3g50910	---	expressed protein
600	245369_at	667.30	4.84	6.11	22.22	At4g15975	At4g15975	---	zinc finger (C3HC4-type RING finger) family protein
603	261651_at	668.93	4.83	4.94	15.22	At1g27760	At1g27760	---	interferon-related developmental regulator family protein / IFRD protein family
605	255599_at	670.65	4.86	6.44	24.61	At4g01010	At4g01010	---	cyclic nucleotide-regulated ion channel, putative (CNGC13)
606	251603_at	671.19	4.85	4.60	12.64	At3g57760	At3g57760	---	protein kinase family protein
609	264767_at	673.66	4.88	5.85	20.74	At1g61380	At1g61380	---	S-locus protein kinase, putative
612	256989_at	676.61	4.91	8.07	37.06	At3g28580	At3g28580	---	AAA-type ATPase family protein
613	249984_at	677.23	4.91	4.46	11.87	At5g18400	At5g18400	---	expressed protein
615	261193_at	681.08	4.95	6.51	25.34	At1g32920	At1g32920	---	expressed protein

4.5 Follow Up Studies on the Significance of *HY5* Gene Expression in Response to UV-B

The microarray data presented above suggests that there is a reduction in UV-B induced *HY5* gene expression in the *uvr8* mutants. We decided to try to confirm this result using RT-PCR and also to look at the susceptibility of the *hy5* mutant to supplementary UV-B induced growth inhibition and leaf damage. Such studies may help us understand the importance of the link between UVR8 and *HY5* gene expression in the response of *Arabidopsis* to UV-B.

4.5.1 *HY5* Gene Expression is Significantly Impaired in *chum31bc2* and *uvr8bc5* Mature Mutant Leaf Tissue in Response to UV-B But Not UV-A, Whilst *HY5* Upregulation by Far-Red Treatment is Unaltered in Mutant Seedlings

In order to examine *HY5* transcript accumulation in *Arabidopsis* using quantitative RT-PCR a number of preliminary studies had to be carried out. First several sets of primers, each corresponding to the *HY5* cDNA sequence, were tested and a pair yielding a good quantity of PCR product (404 bp in size) from total *Arabidopsis* cDNA was identified. Searches for short, nearly exact, nucleotide – nucleotide matches with these *HY5* primer sequences using the NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/blast/) demonstrated that the primers chosen were specific for the *Arabidopsis HY5* cDNA sequence and would not amplify the similar *HYH* transcript. These *HY5* primers together with the previously used *ACTIN2as* (Fontaine et al., 2002) primers (which indicate that equivalent amounts of total mRNA are represented in each lane of a particular gel) were further tested, amplifying template cDNA over a range of cycle numbers. It was found that 24 cycles of PCR represented a stage in the amplification process where DNA production by both sets of primers (*ACTIN2as* and *HY5*) in a single reaction tube was in the logarithmic phase.

As with *CHS*, it was unclear whether UV-B induction of *HY5* gene expression was reduced in *uvr8bc5* / *chum31bc2* etiolated seedlings (Figure 4.21), whilst mature mutant leaf tissue clearly showed little or no increase in *HY5* expression in response to UV-B (Figure 4.22). Each of these experiments was repeated, using a separate batch of plant tissue but the same

treatments, and the results of all four analyses are shown below (Figures 4.21 and 4.22). These data, together with that accumulated on *CHS* expression, suggest that UVR8 is playing an important role in regulating responses to UV-B in mature *Arabidopsis* leaf tissue at least. It should be noted that the microarray data shown above was generated from mature *Arabidopsis* leaf tissue.

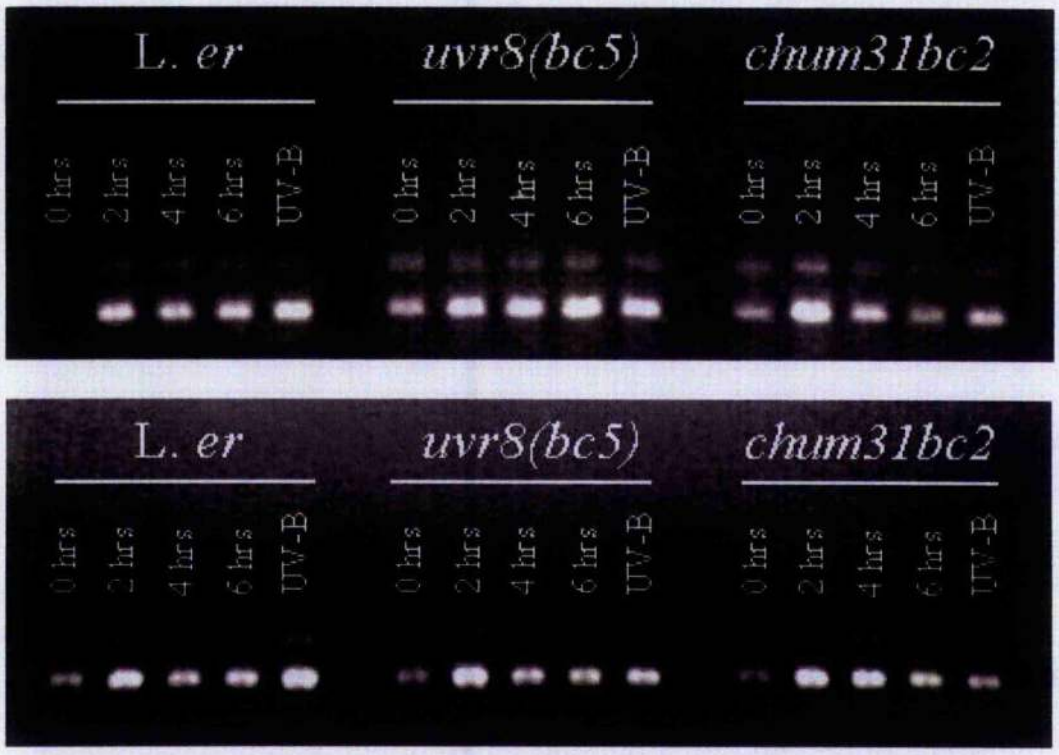


Figure 4.21
Reverse transcriptase (RT)-PCR analysis of *HY5* mRNA induction by UV-B and Far Red light. Four day old, dark grown L. *er* wild-type, *uvr8bc5* and *chum31bc2* (line 1) seedlings (grown on media containing sucrose) were treated with 0, 2, 4 or 6 hours 70 $\mu Em^{-2}s^{-1}$ Far Red light or 4 hours of 3 $\mu Em^{-2}s^{-1}$ UV-B (as indicated). Tissue was then harvested for RNA extraction and ethidium bromide stained products are shown from quantitative RT-PCR depicting *HY5* expression (lower bands) compared to an *ACTIN* loading control (upper bands). The results shown (upper and lower panels) are taken from two separate, but identical experiments.

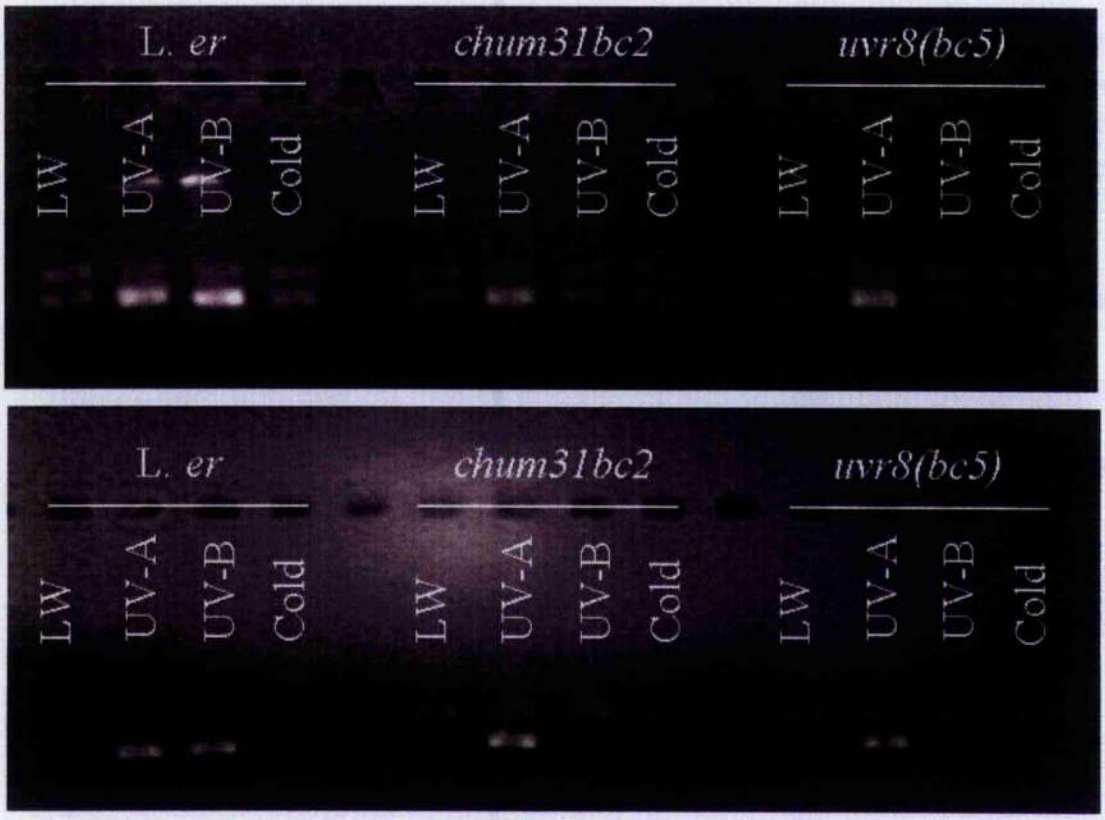


Figure 4.22
Reverse transcriptase (RT)-PCR analysis of *HY5* mRNA induction by UV-A, UV-B and low temperature. L. *er* wild-type, *uvr8bc5* and *chum31bc2* were grown up under low fluence rate white light (18-35 $\mu Em^{-2}s^{-1}$) for 3 weeks before treatment with either 100 $\mu Em^{-2}s^{-1}$ UV-A for 6 hours, 3 $\mu Em^{-2}s^{-1}$ UV-B for 4 hours, 7-10°C in low white light for 24 hours (Cold) or no further treatment (LW). Tissue was then harvested for RNA extraction and ethidium bromide stained products are shown from quantitative RT-PCR depicting *HY5* expression (lower bands) compared to an *ACTIN* loading control (upper bands). The results shown (upper and lower panels) are taken from two separate experiments.

As was the case for *CHS*, it is clear that the expression of *HY5* in response to far-red light in etiolated seedlings (Figure 4.21) and cold treatment in mature leaf (Figure 4.22) is unaltered in the *uvr8* mutants. However, neither far-red nor low temperature appears to induce *HY5* as effectively in wild-type (L. *er*) *Arabidopsis* as the same treatments do *CHS* (Figure 4.16 and Figure 4.17). It therefore seems unlikely that HY5 plays a significant role in the response of *Arabidopsis* plants to cold treatment. There does appear to be increased *HY5* transcript accumulation in response to far-red (Figure 4.21), compared to *HY5* mRNA levels in untreated etiolated seedlings (0 hrs) - an increase seen in both L. *er* and *uvr8* mutants; but

there is no sign of further increase in *HY5* gene expression when seedlings are irradiated with far-red light for durations exceeding 2 hours.

4.5.2 The Increased Susceptibility of the *hy5* Mutant to Supplementary UV-B Induced Growth Inhibition and Leaf Damage is of a Similar Extent to that Seen in the *uvr8* Mutants

The treatment conditions described in Section 4.4.2 were used to investigate the susceptibility of the *hy5* mutant to supplementary UV-B induced growth inhibition and leaf damage. The *hy5* mutant proved to have an enhanced susceptibility to supplementary UV-B and to show a similar level of growth inhibition and leaf damage to that seen in the *uvr8* mutants (Figure 4.23). It seems likely therefore, that the ability of *Arabidopsis* to resist damage caused by high fluence rate supplementary UV-B depends partly upon an intact signal transduction pathway which is routed through both UVR8 and HY5. Since the microarray data presented in Section 4.4.6 demonstrated that expression of a number of flavonoid biosynthesis genes is induced by UV-B *via* UVR8 and since expression of some of these genes, eg. *CHS* (Wade and Jenkins, unpublished work), is induced by UV-B *via* HY5 also, it seems likely that UV-B stimulates the production of UV-B protective, flavonoid-based sunscreens by way of a signalling pathway in which both UVR8 and HY5 play an integral part. Interestingly, previous work using HPLC had established that whilst the mutant accumulates 50% less total flavonoids than does a wild-type plant following UV-B treatment, sinapate ester induction occurs normally in *uvr8* (Kliebenstein et al., 2002). It is also noteworthy that a number of other genes which could have important roles to play in UV-B photoprotection were identified in Section 4.4.6 as UVR8 regulated. A second series of microarray studies has recently shown that many of these genes are also induced by UV-B *via* HY5. A brief description of these recent findings is contained in Section 7.5.

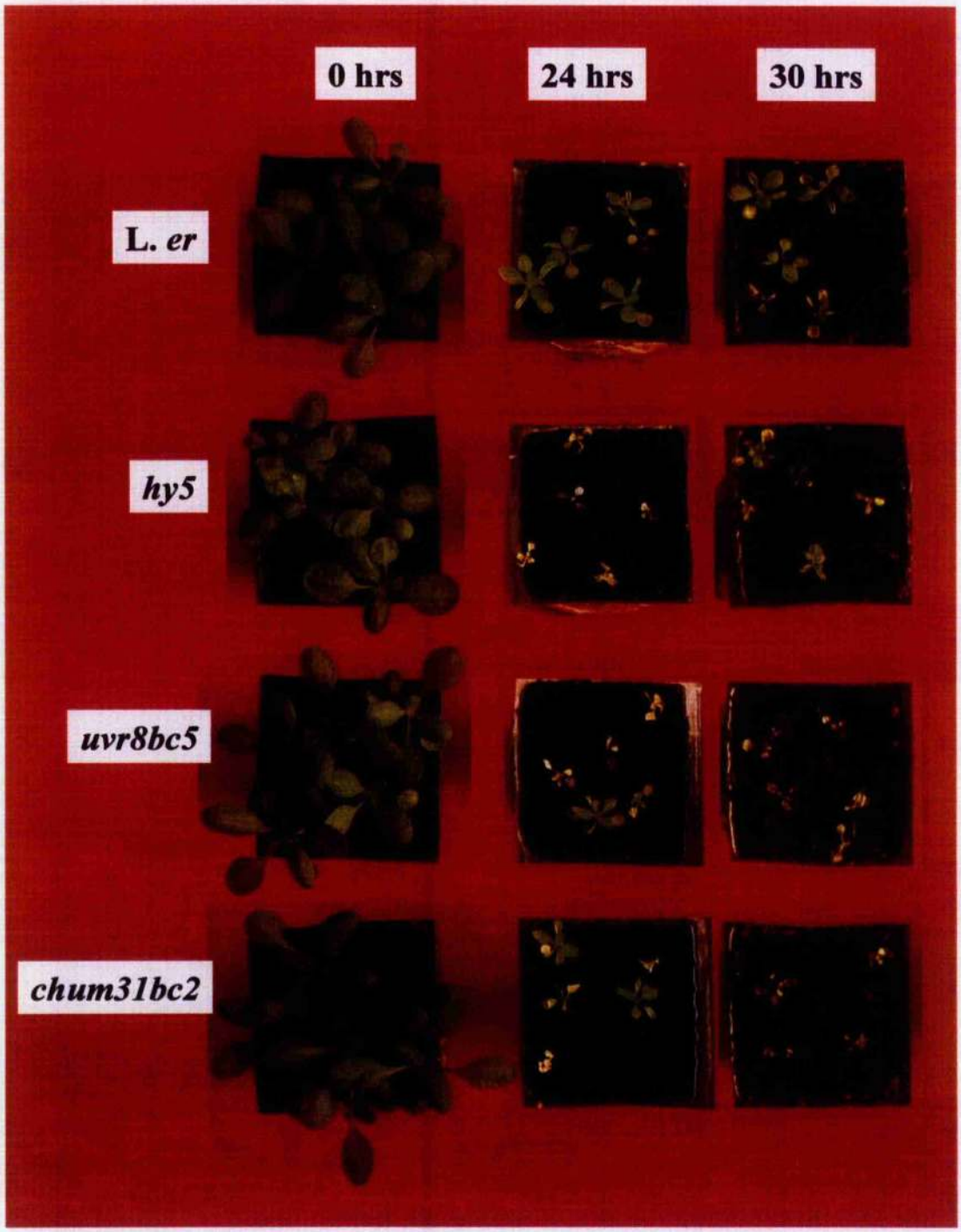


Figure 4.23
The *hy5* and *uvr8* mutants are equally susceptible to supplementary UV-B induced tissue damage. Wild-type L. *er*, *hy5*, *uvr8bc5* and *chum31bc2* seedlings were grown up under 120 $\mu Em^{-2}s^{-1}$ white light for 12 days before treatments of varying durations (as indicated) with 5 $\mu Em^{-2}s^{-1}$ UV-B (Supplemented with 40 $\mu Em^{-2}s^{-1}$ white light). The pictures shown were taken after a 5 day recovery period in 120 $\mu Em^{-2}s^{-1}$ white light.

4.6 Discussion

4.6.1 The Approach to Isolating Mutants Altered in UV-B Induced *CHS* Expression was a Key Factor in the Success of the Screen

A good deal of thought preceded the search for mutants altered in UV-B induced *CHS* gene expression and many factors were considered to try to optimize the screen and make it as practical as possible to perform. The luciferase reporter gene system has previously proved successful in other mutant screens (Michelet and Chua, 1996, Xiong et al., 1999) and pilot experiments allowed us to decide upon the best plant growth and UV-B treatment conditions for screening a large number of seedlings effectively (see Section 4.2.1.1). Since *Arabidopsis* has around 26,000 genes and only about 7,000 M_1 plants were generated here, there certainly could be scope for genes playing a role in UV-B induced *CHS* gene expression to have gone unnoticed. However, it is difficult to know how many gene mutations each M_1 plant is likely to have and the fact that 4 mutant alleles of the same (*UVR8*) gene were identified suggests that the screen has been quite successful in covering the genome. If there are additional proteins which play a role in UV-B induced *CHS* gene expression in *Arabidopsis* but whose functions are shared, in a redundant manner, by other gene products the corresponding genes would not have been detectable.

It is a little puzzling that only four mutants having reduced *CHS* expression in response to UV-B were finally selected after transcript accumulation was examined, whilst 113 putative underexpressors were identified at the luciferase stage of the screen. Some of these putative mutants may have been altered only in luciferase transgene expression or perhaps in some cellular component which allows the luciferin substrate to penetrate plant tissue more easily or the luciferase to luminesce more brightly. It is also likely that the RT-PCR stage of the mutant screen was conducted more rigorously than the luciferase stage, resulting in many putative mutants which showed only slight alterations in *CHS* expression levels being discarded. More difficult to explain is why one putative mutant, *chum35*, initially selected as an underexpressor was finally described as an overexpressor of UV-B induced *CHS* gene

expression. Perhaps with such an abundance of false positive underexpressors, it is not entirely surprising that one should display the characteristics of an overexpressor when studied more carefully.

4.6.2 Mutants Altered Specifically in UV-B Perception or Signalling *en route* to *CHS* Gene Expression were Isolated from the Screen

Although it appears that both underexpressing and overexpressing mutants altered specifically in the response to UV-B illumination have been isolated, the primary focus of our screen was finding mutants with reduced levels of *CHS* expression because these may lead to the discovery of positive regulators of the UV-B signalling pathway. Perhaps the most significant finding was that four independently generated mutants, altered in a single gene (*UVR8*), were the sole products of an extensive screen for deficiencies in UV-B induced *CHS* transcript accumulation. As a result, whilst a mutant screen may be unable to identify proteins which share redundant functions in a pathway, it seems likely that the UV-B specific part of the *CHS* gene expression pathway is extremely short – perhaps consisting of only a few proteins or even of only UVR8. The UV-B specific role of the UVR8 protein gives it a rare and possibly unique status in the photoperception / signalling pathways of higher plants.

Sequencing of the mutant alleles has confirmed their identity and provided clues which may enable us to understand which parts of the 440 amino acid polypeptide sequence play critical roles in UVR8 function (see Section 4.4.1). Based on DNA sequence information, the *chum31* mutant (hereafter designated *uvr8-2*) should produce a truncated UVR8 protein (Cloix, Jiang and Jenkins, unpublished work) which appears to be completely non-functional, despite losing only 40 amino acids from the carboxyl end. The substitution of a glycine residue found in the middle of the *chum75* (*uvr8-5*) UVR8 polypeptide, with a much larger and acidic glutamate also has a severe effect on UV-B induced *CHS* induction. Since glycine with only a single hydrogen atom side chain plays an important role in allowing unusual main-chain conformations in many proteins (Branden and Tooze, 1999) it is quite likely that a disruption

of UVR8 secondary structure in *chum75*, rather than a direct effect on any catalytic function, is responsible for reduced *CHS* expression when Gly-283 is replaced.

Although as previously described, the UV-B induced *CHS* expression signalling pathway is separate from that responsive to UV-A (Jenkins et al., 2001), the two apparently share components such as HY5 (Wade, 1999). It is somewhat surprising therefore, that mutants deficient in both UV-A and UV-B induced *CHS* gene expression did not emerge during the luciferase stage of the UV-B screen. Perhaps the fact that the *hy5* mutant was not isolated here is explicable by a functional redundancy amongst transcription factors with overlapping roles in UV-B induced *CHS* gene expression, such as HY5 and possibly HYH (Wade and Jenkins, unpublished work). This hypothesis is consistent with the data from Table 4.19 which indicates that UV-B induced transcript accumulation of both the *HY5* and *HYH* genes is mediated by UVR8. Thus the loss of UVR8 could prevent either HY5 or HYH accumulating in response to UV-B and a bigger reduction in *CHS* gene expression would be observed than in a mutant deficient in only one of these transcription factors.

The UVR8 protein appears to act specifically to promote UV-B induced expression of a subset of genes. Accordingly, expression of the *CHS* and *HY5* genes is altered very little in the *uvr8* mutant in response to UV-A or cold treatment (both studied in mature leaf tissue), and to far-red light (studied in seedlings). The *uvr8* mutation does not alter tannin build-up in *Arabidopsis* seeds, nor does it change the level of anthocyanin accumulation in response to treatment with methyl jasmonate (Kliebenstein et al., 2002). In fact, to date no clear role has been shown for UVR8 in the regulation of anything other than UV-B induced responses, although a small reduction in UV-A induced *CHS* expression has been observed (see Figure 4.16). Conversely, UVR8 clearly does have an important role in mediating UV-B stimulated *CHS* and *HY5* transcript accumulation in mature *Arabidopsis* leaf tissue. Additionally, Kliebenstein *et. al* showed that the *uvr8* mutation visibly reduced UV-B elicited anthocyanin accumulation in seedling hypocotyls (Kliebenstein et al., 2002) - although it is worth noting that our data was less clear on whether UVR8 plays as significant a role in regulating the UV-

B responses of etiolated seedlings (see Section 4.4.5 and 4.5.1) as it does in controlling those of mature leaf.

Other than *uvr8*, there are very few mutants which appear to be deficient solely in UV-B responses. One of the few *Arabidopsis* mutants which may be altered only under UV-B is the *uli3* mutant (Suesslin and Frohnmeyer, 2003). However, alterations in *CHS* expression and inhibition of hypocotyl elongation in *uli3* mutant seedlings were observed in response to a combination of UV-A and UV-B wavelengths and not to UV-B alone. A more rigorous examination of the ULI3 gene product will be required to establish its UV-B specific credentials.

In addition to the underexpressing mutants, two mutants overexpressing *CHS* in response to UV-B were isolated as a result of the screen described herein. These mutants, *chum2* and *chum35*, may well be altered specifically in their responses to UV-B and could be the basis of some interesting follow-up work.

4.6.3 The UVR8 Gene Product Has a Key Role to Play in Protecting *Arabidopsis* from Damaging UV-B

A salient feature of the group of genes found in Table 4.19, which describes those induced by UV-B *via* UVR8, is that many may have a protective function (see Section 4.4.6.2). For example, upregulation of the flavonoid / phenylpropanoid biosynthesis genes identified (*F3H, CHS, FLS1, CHI, DFR* and *4CL3*) might result in enhanced production of epidermal UV protectants or antimicrobial agents (Sakuta, 2000, Winkel-Shirley, 2001). Early light-induced proteins (ELIPs), such as those which head the list of UV-B induced genes regulated by UVR8, also appear to play a photoprotective role in *Arabidopsis* since suppressing their accumulation whilst plants are treated with high fluence rate light can result in bleaching of leaves and photooxidative damage (Hutin et al., 2003).

The microarray results show that genes which might be important in combating oxidative stress are found amongst both the UVR8-regulated and non UVR8-regulated, UV-B induced genes. However, two putative glutathione peroxidases and two putative FtsH proteases are

each found only amongst the UVR8-regulated, UV-B induced genes (Table 4.19). Whilst it is important to remember that the precise function of these gene products has not been clearly established, it seems likely that they too are part of a group of UVR8-regulated genes which protect the plant against some of the deleterious effects of low wavelength light. There is no question that the type II CPD photolyase gene *PHR1* (Sakamoto et al., 1998), found exclusively in Table 4.19, also belongs in this category as it repairs UV-B induced lesions formed in the DNA.

The consequences of losing the capacity for UVR8 induced upregulation of genes such as those described above may be seen in Figures 4.14 and 4.23 wherein a reduction in tolerance of supplementary UV-B light treatment is evident in the *uvr8* and *hy5* mutants.

Further work will be required to answer some of the questions raised by the data in Tables 4.19 and 4.20. For example, why is it that UVR8 is implicated in upregulating the recently discovered putative photoreceptor cryD / cry3 (Kleine et al., 2003)?

Also significant is the fact that a great many UV-B induced genes appear to be regulated entirely independently of UVR8 (Table 4.20). Clearly there is more to UV-B photoperception than UVR8. Identifying large classes of genes induced by UV-B in the absence of UVR8 (ie. those classes found in Table 4.20 but not Table 4.19) is surprisingly difficult however. One such class is the MATE efflux family (related) proteins of which at least eight occur on the UVR8 independent, UV-B regulated gene list. These multidrug and toxic compound extrusion (MATE) family proteins are similar to bacterial efflux transporters and may perform similar roles in plants. *Arabidopsis* has at least 54 members of this MATE family but they are not found at all in the animal kingdom (Diener et al., 2001). One MATE efflux family protein which is highlighted by Table 4.20 is EDS5 (enhanced disease susceptibility 5) or SID1 (salicylic acid induction deficient 1). The *Arabidopsis eds5* mutant shows an increased susceptibility to pathogens aud a reduced response to infection (less salicylic acid and PR-1 transcript accumulation). The *EDS5* gene is expressed strongly in response to pathogens or UV-C light in wild-type plants. Related work has shown an overlap between the signalling components involved in UV-C and those taking part in the pathogen induced pathways

(Nawrath et al., 2002). It would appear from our work, that these particular pathways which lead to *EDS5* induction, and the expression of other MATE efflux family protein genes, are likely to be distinct from those UV-B induced pathways controlled by UVR8.

A group of transcription factors, comprising no less than nine WRKY family proteins, also occurs in Table 4.20 but not Table 4.19. These WRKY transcription factors are part of a major family which are essential in pathogen and salicylic acid responses of higher plants (Yamasaki et al., 2005) and it now appears that they can also regulate UV-B responses independently of UVR8. There is evidence, however, that UVR8 also exerts some of its effects *via* transcription factors, especially the bZIP transcription factors HYH and HY5 (Table 4.19).

4.6.4 HY5 is an Important Effecter of UV-B Response(s) Controlled by UVR8

It is clear from the microarray results (Table 4.19) that UVR8 mediates UV-B induced upregulation of genes encoding the transcription factors HY5 and HYH. These bZIP transcription factors have partially overlapping functions in *Arabidopsis* gene expression and development and promote photomorphogenesis in light grown plants (Holm et al., 2002, Schwechheimer et al., 2002). Each appears to be degraded in the dark in the presence of negative regulators of photomorphogenesis, including COP1 and the COP9 signalosome (CSN).

HY5 is one of the most important regulators of light responses in *Arabidopsis*. It plays a general role promoting photomorphogenesis in seedlings (Andersson and Kay, 1998, Hardtke et al., 2000, Osterlund et al., 2000) and a more specific role mediating gene expression in response to far-red, UV-A / blue and UV-B illumination (Ang et al., 1998, Wade, 1999, Ulm et al., 2004). As described in Chapter One, HY5 is a basic leucine zipper (bZIP) transcription factor which binds directly to the promoters of light-inducible genes (Osterlund et al., 2000) and illumination increases its nuclear abundance (Hardtke et al., 2000). HY5 is stabilized by a light-regulated kinase activity but whilst phosphorylation renders the protein less susceptible to COP1 mediated degradation, it also may reduce HY5's affinity for target promoters. A

small pool of less reactive, phosphorylated HY5 could be maintained in dark grown plants to produce a rapid reaction to illumination (Hardtke et al., 2000).

Most of the previous studies investigating the regulation of HY5 activity have concentrated on its interaction with COP1 and subsequent degradation at the protein level (Hardtke et al., 2000, Holm et al., 2001). We have shown that expression of the *HY5* gene is increased on illumination and that UV-B induced *HY5* upregulation depends on UVR8 in mature leaf tissue (Table 4.19 and Figure 4.22). To our knowledge, UVR8 is the first protein which has been shown to regulate HY5 at the mRNA level in mature leaf tissue. Since it is involved in blue, red and far-red induced responses together with basic plant developmental processes, HY5 is believed to be downstream of convergence points between signals from multiple photoreceptors and other developmental cues (Hudson, 2000). It has been demonstrated that HY5 is required for UV-A / blue and also UV-B induction of *CHS* gene expression in mature leaf tissue (Wade and Jenkins, unpublished work). It has been shown here that UV-A and UV-B each stimulate *HY5* (Figure 4.22) and *CHS* (Figure 4.16) transcript accumulation but that only the UV-B pathway(s) require UVR8. It seems likely that UV-B induces *CHS* expression *via* UVR8 and then HY5. It will be intriguing to discover whether UV-A induction of *HY5* gene expression is lost in the *cry1* and *cry1cry2* double mutants.

Following on from the work on UVR8, we were interested in discovering whether HY5 mediates UVR8 regulated expression of some of the other UV-B induced genes found in Table 4.19. Consequently, a second microarray experiment focusing on finding UV-B responses regulated *via* HY5 has recently been completed. As shown in Table 4.19, 79 UV-B induced genes appear to require UVR8, although 6 of these genes (highlighted in Table 4.19) also fit the criteria for being independent of UVR8. The recent *hy5* results have indicated that 68 of the 645 genes found to be induced in response to UV-B require the presence of HY5. A total of 37 genes appear on both (UVR8 and HY5 dependent) lists and therefore constitute a subset of genes which are almost certainly induced by a UV-B signal which is transmitted *via* UVR8 mediated, *HY5* transcript accumulation (see Section 7.5 for a brief discussion of these

genes). Note that all of the microarray data discussed in this work is subject to cut-off at a false discovery rate (FDR) of 5%.

Examining the roles of some of the other transcription factors expressed in response to UV-B either *via* UVR8 (such as HYH) or independently of UVR8 (such as the WRKY transcription factors) will allow a more comprehensive dissection of UV-B signalling pathways in *Arabidopsis thaliana*.

4.6.5 The Mechanism by Which UVR8 Induces Responses to UV-B in *Arabidopsis* is Unclear

The positive regulator of UV-B responses UVR8 clearly plays a very significant role in *Arabidopsis*, upregulating the expression of a number of important genes including *HY5*. However, details of the mechanism by which UVR8 mediates UV-B induced gene expression are still to be uncovered. It is certain that UVR8 is involved either in UV-B photoreception or signal transduction. A lack of obvious transcription factor structural motifs suggested by the UVR8 polypeptide sequence together with the paucity of other mutants isolated in our UV-B screen might indicate that UVR8 plays a direct role in photoreception. However, the RCC1 (regulator of chromatin condensation) protein with which UVR8 shares significant sequence identity (see below) interacts with histones H2A and H2B (Nemergut et al., 2001), which suggests UVR8 might regulate transcription through an association with chromatin. In addition, the fact that a UVR8 fusion protein expressed in *E. coli* failed to exhibit a significant level of UV-B absorption (Cloix and Jenkins, unpublished work), combined with a lack of obvious binding sites for flavin or pterin chromophores suggested that signal transduction rather than photoreception was the more likely function of UVR8. It is worth noting, however, that the phot1 photoreceptor LOV domain was originally described as having "little or no sequence similarity with known flavin-binding sites" (Huala et al., 1997), so the possibility that UVR8 is also a flavoprotein photoreceptor cannot be ruled out.

Whilst it is conceivable that there is functional redundancy amongst an elusive group of photoreceptors, it is possible that UVR8 is the first proteinaceous component in UV-B

phototransduction *en route* to *CHS* gene expression. If so, one alternative to the foregoing suggestions is that the UVR8 protein acts as a sensor, detecting a disturbance amongst cellular components disrupted under UV-B illumination. In this case, perhaps impairment of photosynthesis, break-up of lipid membranes or formation of pyrimidine dimers in the DNA is the photoperception event. As described in Chapter One (Section 1.3.3.4.3), there is separate experimental evidence consistent and inconsistent respectively with the hypothesis that damage to DNA induces *CHS* expression. Therefore, because UV-B can induce gene expression in animal cells *via* DNA damage (Bender et al., 1997) and also because UVR8 associates with chromatin in *Arabidopsis* (Cloix and Jenkins, unpublished work), we investigated the possibility that UV-C (which should generate damage to DNA) may stimulate *CHS* expression *via* UVR8. However, no good evidence that our UV-C light source upregulates *CHS* at all in either mature leaf or etiolated wild-type L. *er* seedlings was found. Corroboration that our 255 nm UV-C source (DNA absorbs light maximally at 260 nm) did indeed produce damage to nucleic acids in mature leaf tissue came from the fact that at high doses of UV-C it was uncharacteristically difficult to amplify even the *ACTIN* control PCR product using cDNA derived from irradiated tissue (data not shown). Hence, it seems unlikely that *CHS* expression is stimulated by a DNA damage signalling pathway.

In order to try to understand how UVR8 operates *in vivo*, sequence comparisons have been explored. The predicted UVR8 protein is similar (35% identical and 50% similar) to the Regulator of Chromatin Condensation 1 (RCC1) family of proteins which are guanine nucleotide exchange factors (GEF) for the small G-protein Ran (Kliebenstein et al., 2002). RCC1 occurs in a variety of organisms and, in concert with Ran, regulates processes as diverse as the cell cycle and transport across the nuclear membrane (Clark et al., 1991, Demeter et al., 1995, Seki et al., 1996, Li et al., 2003). The degree of functional similarity shared by UVR8 and RCC1 is currently being assessed. Whilst UVR8, like RCC1, is capable of binding histones, it appears to have little appreciable Ran GEF activity (Cloix and Jenkins, unpublished work) and doesn't interact directly with Ran (D. J. Kliebenstein, personal communication). Interestingly, mammalian RCC1 contains a nuclear localization signal

(NLS) not present in the UVR8 protein. However, RCC1 can migrate into the nucleus even without its NLS (Nemergut and Macara, 2000). Unpublished work shows that UVR8 (attached to Green Fluorescent Protein) accumulates in the nucleus following UV-B illumination (Jiang, Kaiserli and Jenkins, unpublished work). Presently, it is not clear whether this accumulation is due to translocation into the nucleus, inhibition of degradation or both.

In many respects it seems the differences between UVR8 and RCC1 are likely to be more significant than the similarities. For example, the UVR8 protein contains a peptide loop which is not found in RCC1 but could constitute part of a separate functional domain (Kaiserli, Cloix and Jenkins, unpublished work). The contrasts in sequence and function suggest that UVR8 may play a role in higher plants which is unrelated to that fulfilled by RCC1. Support for this interpretation, proposing a lack of UVR8 involvement in cell cycle regulation or nucleocytoplasmic trafficking, is drawn from the lack of any evidence of a deleterious *uvr8* mutant phenotype in the absence of UV-B (see *chum31*, *chum33*, *chum53* and *chum75* mutants in Figure 4.10). By contrast, *rcc1* mutations, documented in a variety of species, tend to have serious and often fatal consequences (Aebi et al., 1990, Kadowaki et al., 1993, Matsumoto and Beach, 1991, Sazer and Nurse, 1994, Kliebenstein et al., 2002).

Elucidating the mechanism of UVR8 action certainly requires further work but it is anticipated that the interaction with chromatin is an important observation. The possibility that UVR8 is somehow involved in the formation of a DNA-bound transcription factor complex which promotes the expression of UV-B inducible genes is one that should be considered. There are examples of gene expression being controlled directly by chromatin modification. For example, histone acetylation is one of the best studied covalent modifications of core histones and is associated with transcriptional activation in all eukaryotes (Kuo and Allis, 1998, Brownell et al., 1997, Mizzen et al., 1998). Selective acetylation of histone protein tails creates space between nucleosome particles, generating local areas of chromatin decondensation which are more accessible to transcription factors and hence more amenable to gene expression (Buchanan et al., 2000). Perhaps herein lies the

significance of the sequence similarity shared by UVR8 and RCC1 (regulator of chromatin condensation).

Just as illumination appears to activate UVR8, so should future work shed light on the dynamics of its activation. Undoubtedly UVR8 is a key component regulating the expression of a subset of critical UV-B induced genes in *Arabidopsis thaliana*.

CHAPTER 5

A SCREEN FOR MUTANTS DEFICIENT IN AN ULTRAVIOLET-B INDUCED POSITIVE PHOTOTROPIC HYPOCOTYL RESPONSE

5.1 Introduction

Phototropism is the process by which plants reorient the growth of organs, in particular stems or hypocotyls, in response to lateral differences in light quality or quantity (Liscum and Stowe-Evans, 2000). Phototropic hypocotyl curvature (shown in Figure 5.1) in response to UV-A / blue wavelengths is mediated by the phototropin photoreceptors phot1 and phot2 (Briggs and Christie, 2002). UV-B can stimulate phototropic curvature too (see Figure 5.2 which illustrates the alfalfa phototropism action spectrum) and some of this curvature may be attributable to the phototropins. However, it is also possible that UV-B induced phototropic curvature in plants such as *Arabidopsis thaliana* may be mediated, in whole or in part, by a separate UV-B specific photoreception system. Here, evidence is presented which indicates that the *Arabidopsis phot1-5phot2-1* double mutant displays positive phototropic hypocotyl curvature towards a UV-B light source. When UV-B wavelengths are removed from the source by a filter, curvature is still observed (whereas dark grown, unilluminated seedlings grow directly upwards) but is no longer as consistently directed towards the light. This data shows that UV-B wavelengths can induce a positive phototropic response in *Arabidopsis thaliana* even in the absence of the two known phototropic photoreceptors, phot1 and phot2. An extensive screen was carried out in the *phot1-5phot2-1* double mutant background to isolate mutants which had lost the UV-B specific phototropic response. However, after rigorous examination of putative mutants, it was concluded that no true mutants had been found which were consistently deficient in the UV-B induced phototropic hypocotyl response.

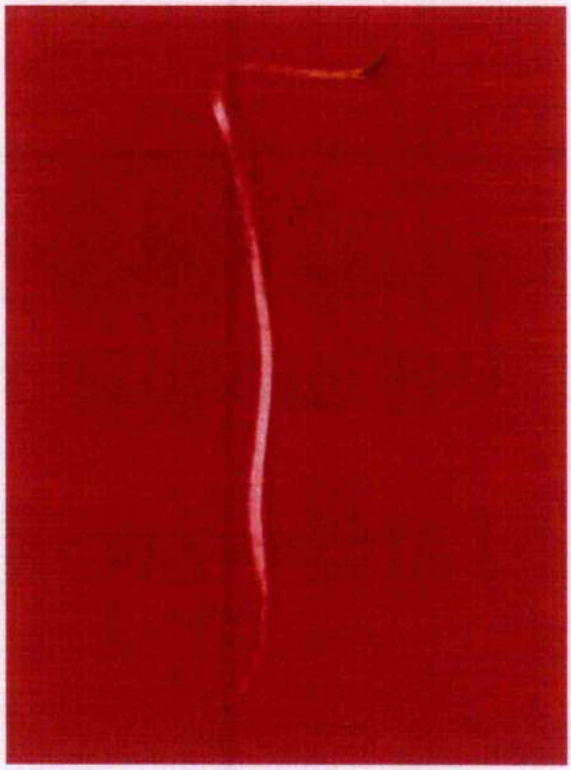


Figure 5.1
Positive phototropic hypocotyl curvature. An etiolated *Arabidopsis thaliana* seedling bends towards a light source (located to the right of the picture). The seedling shown is a *phot1-5phot2-1* double mutant and the incident light approximately 0.5 $\mu Em^{-2}s^{-1}$ UV-B. Such conditions were used for screening.

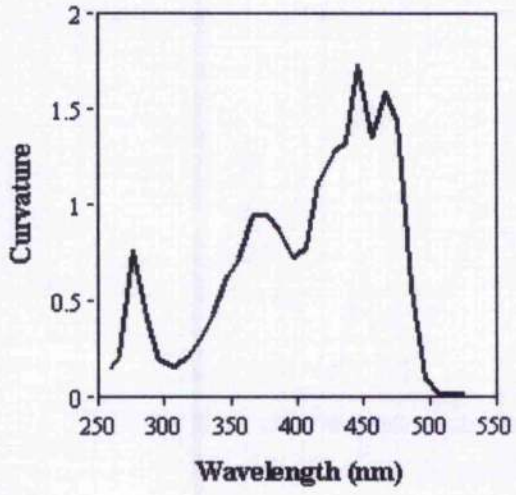


Figure 5.2
Action spectrum for alfalfa phototropism. Curvature can be induced by UV-B light (280-320 nm) in alfalfa (Taken from Baskin and Iino, 1987).

5.2 It was Suggested that if UV-B Induced Hypocotyl Curvature could be Observed in a Mutant Lacking the Two Known Phototropin Photoreceptors, then this *phot1phot2* Double Mutant Could be Used as the Basis of a Screen to Isolate Mutants Deficient in UV-B Induced Phototropism

It was suggested that we investigate whether phototropic hypocotyl curvature could be induced by UV-B in the *phot1-5phot2-1* double mutant (J. M. Christie and W. R. Briggs, personal communication). Because the *phot1-5phot2-1* mutant produces no active phot1 (Huala et al., 1997, Kinoshita et al., 2001) or phot2 (Kagawa et al., 2001, Kinoshita et al., 2001) protein it contains neither of the functioning phototropin photoreceptors which are known to mediate phototropic curvature. Thus if a response is observed in the *phot1-5phot2-1* mutant this cannot be attributed to phot1 or phot2 and must be due to a novel photosensory system (ie. a third pathway). Furthermore, mutants of any response, such as UV-B induced phototropism, still present in the *phot1-5phot2-1* mutant could be generated by mutagenizing the double mutant as the starting point for a screen. In this way, interference by the previously characterized phototropins (phot1 and phot2) could safely be excluded from consideration during the work presently described.

5.3 It was Found that *phot1-5phot2-1* Mutant Seedlings Exhibited a Stronger Phototropic Response to Low Fluence Rate UV-B than to an Almost Identical Light Treatment Which Lacked the UV-B Wavelengths

Of the various light intensities tested, it was found that low fluence rate (≤ 1 $\mu Em^{-2}s^{-1}$) UV-B induced hypocotyl curvature in the *phot1-5phot2-1* mutant more effectively than did high fluence rate (> 1 $\mu Em^{-2}s^{-1}$) UV-B (data not shown). However, as described in Chapter Three, the partially covered UV-B 313 fluorescent tubes used emit a variety of wavelengths (see Chapter Three, Section 3.2.1) and it was important to ensure that UV-B, rather than the other light qualities, was responsible for seedling curvature. To demonstrate that the phototropic response observed was dependant on UV-B wavelengths, a 'Clear 130' filter (Lee Filters, Andover) was used in control 'UV-B Minus' treatments to remove the UV-B wavelengths whilst UV-A, blue, far red, etc. wavelengths present in the source were transmitted (Figure 3.1). It was shown (Figure 5.3) that 'UV-B Plus' (containing UV-B) treatments induced greater or more consistent positive phototropic curvature in *phot1-5phot2-1* mutant seedlings than did illumination with 'UV-B Minus' (lacking the UV-B wavelengths). Efforts were made to try to ensure that equivalent (or slightly greater) fluence rates of other wavelengths (especially UV-A / blue which can induce curvature in wild-type seedlings) were provided in the 'UV-B Minus' treatments. Such a strategy was designed to confirm that seedling curvature observed in response to 'UV-B Plus' treatments is not explicable by higher fluence rates of non - UV-B wavelengths. Since cellulose acetate filters were employed in these experiments the effects of UV-C can also be discounted.

These observations demonstrated that a photoperception system other than either phot1 or phot2 is capable of mediating a UV-B specific phototropic hypocotyl response (although a recent study concluded that UV-B induced *Arabidopsis* hypocotyl bending is mediated by phototropins (Eisinger et al., 2003)). It was therefore decided to try to establish a procedure for generating mutants deficient in UV-B induced positive phototropic hypocotyl curvature.

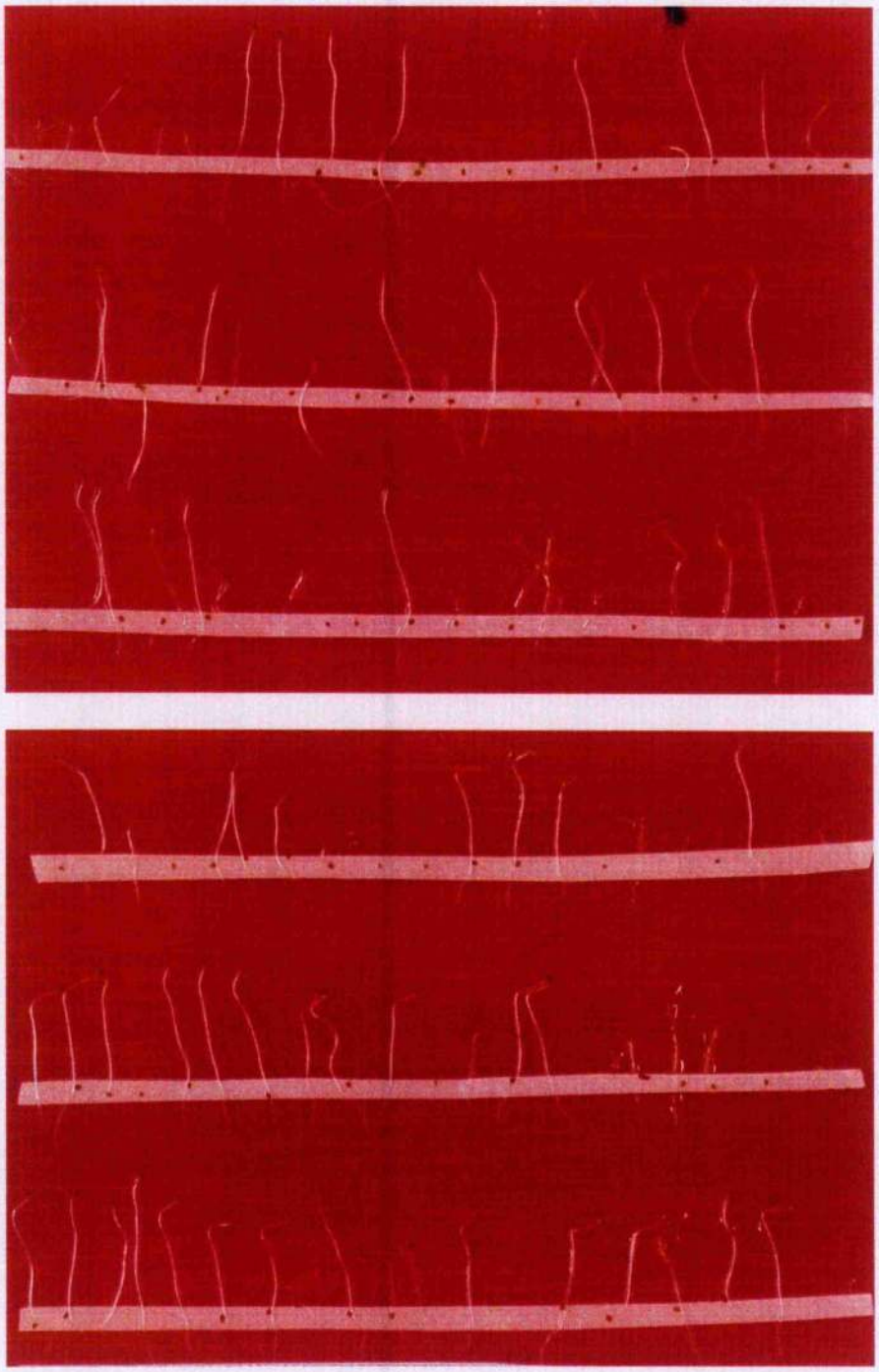


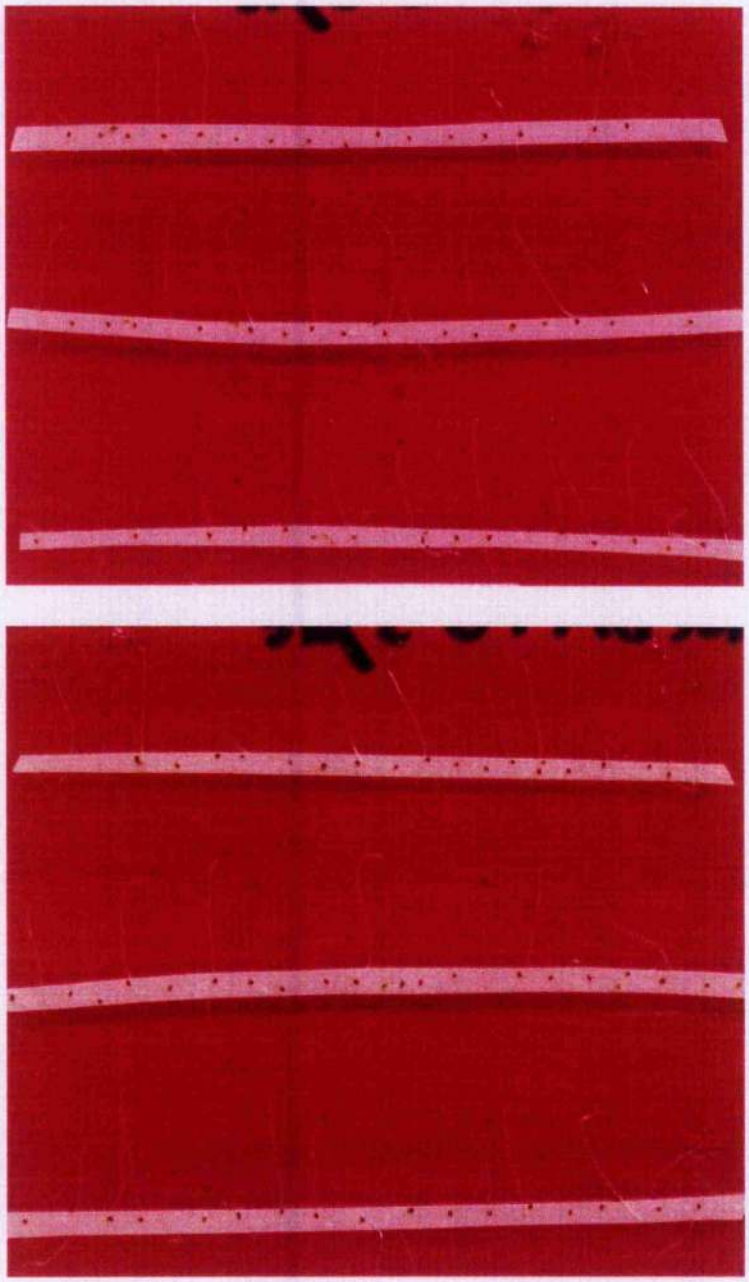
Figure 5.3
UV-B wavelengths specifically can induce positive phototropic hypocotyl curvature in *phot1-5phot2-1* mutant seedlings. Etiolated *Arabidopsis phot1-5phot2-1* double mutant seedlings (grown in darkness for just over 72 hours) were illuminated from the right hand side and for 24 hours with approximately 0.5 $\mu Em^{-2}s^{-1}$ UV-B (lower panel) or with a similar light treatment which lacked the UV-B wavelengths (upper panel). Directed curvature is clearly visible in the UV-B treated seedlings (lower panel) only. The seedlings were grown on media lacking sucrose.

5.4 Either the *phot1-5* or *phot1-5phot2-1* Mutant Could Have Been Used as the Basis of a Screen for Mutants Lacking UV-B Induced Positive Phototropic Hypocotyl Curvature

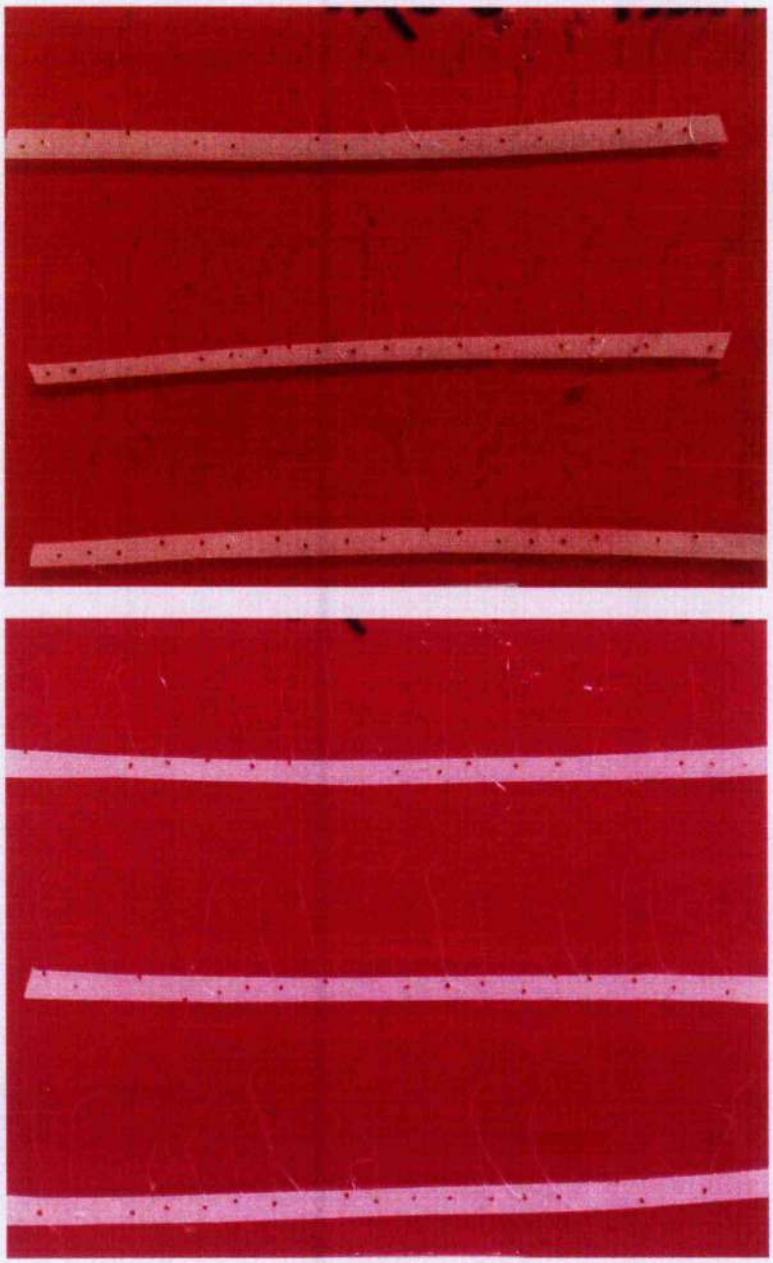
Since wild-type seedlings (containing functional phot1 and phot2 photoreceptors) exhibited a positive phototropic response to non-UV-B light qualities in the 'UV-B Minus' treatments described in the previous section, it would not have been possible to use mutagenized or tagged wild-type seedlings directly as the starting point for the UV-B screen being considered here. In addition, the *phot2-1* mutant (containing functional phot1 only) was found to display a similarly wild-type phototropic response to 'UV-B Minus' whereas the *phot1-5* mutant (containing functional phot2 only) did not. The response of the *phot1-5* mutant was in fact very similar to that of the *phot1-5phot2-1* double mutant (Figure 5.4). Thus, it seems that only phot1 must be removed to demonstrate the existence of a UV-B specific phototropic response. Perhaps the reason that it is not necessary to remove the phot2 photoreceptor is that phot2 is not present in etiolated *Arabidopsis* seedlings until induced by phyA (J. M. Christie, personal communication) Consequently, either the *phot1-5* single mutant or *phot1-5phot2-1* double mutant (which was in fact used) could have been chosen as the basis for our UV-B screen.

Figure 5.4
Only the phot1 photoreceptor must be removed to see UV-B specific phototropic hypocotyl curvature. Etiolated *Arabidopsis* seedlings (grown in darkness for just over 72 hours) were illuminated from the right hand side and for 24 hours with approximately 0.5 $\mu Em^{-2}s^{-1}$ UV-B (lower panels) or with a similar light treatment which lacked the UV-B wavelengths (upper panels). Directed curvature is visible in UV-B treated seedlings (lower panels) of all genotypes, but is absent in mutants of phot1 where UV-B wavelengths have been removed (ie. the upper panels showing *phot1-5phot2-1* and *phot1-5*). The seedlings were grown on media lacking sucrose.

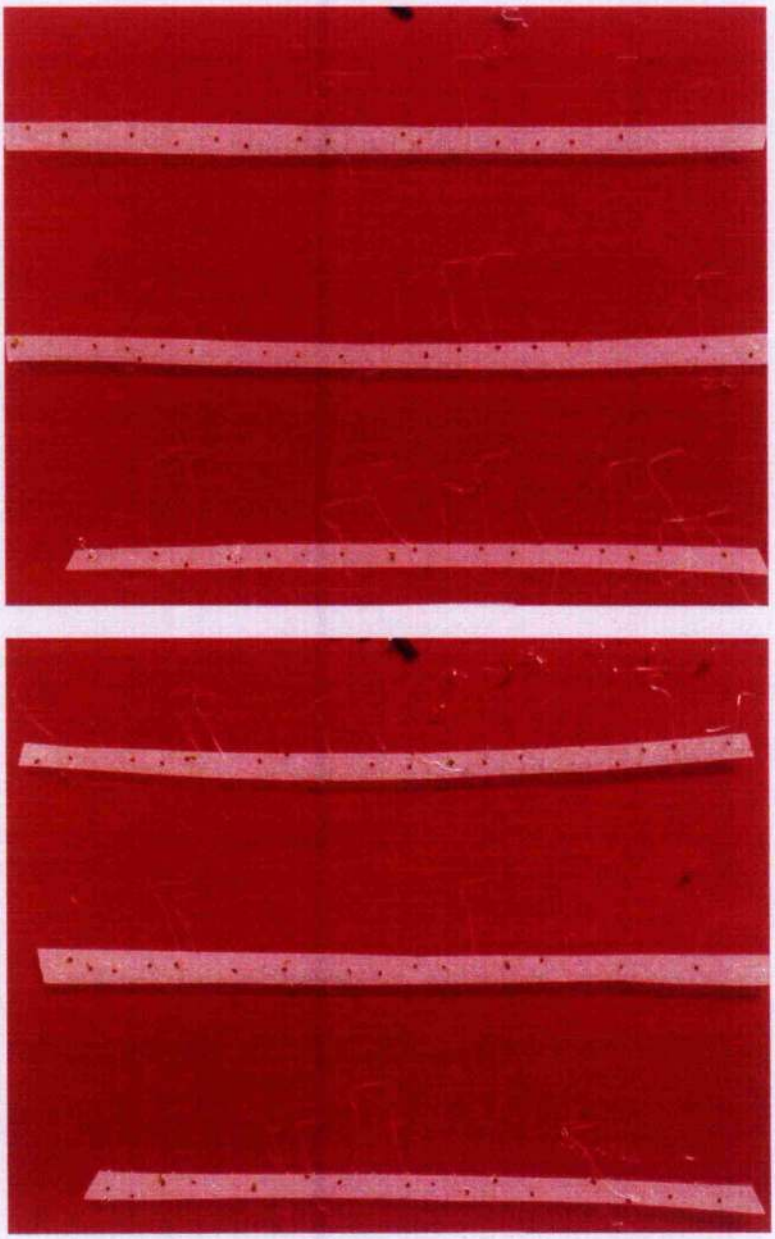
phot1-5phot2-1



phot1-5



phot2-1



5.5 The Present Screen has the Power to Isolate Novel Mutants

Other than those with alterations in the phot1 and phot2 photoreceptors, very few mutants deficient in phototropic hypocotyl curvature have been isolated (Inada et al., 2004), and none which have lost a response to UV-B only. Nevertheless, it is important to consider the possibility that previously identified photoreceptors and / or signalling components may play a role in UV-B stimulated phototropic curvature.

Interestingly, as with *phot2-1* mutant seedlings, mutants of the positive regulator of photomorphogenesis HY5 also retain curvature in response to the 'UV-B Minus' treatment previously described. This suggests that HY5 is not acting downstream of phot1 or a UV-B specific photoreceptor in phototropism (although the possibility that HY5 is acting redundantly, or separately in the UV-B pathway, cannot be excluded). In addition, it is unlikely that phytochrome functions as a primary photoreceptor here because neither red nor far-red light induces phototropism in etiolated seedlings of most species, including *Arabidopsis* (Liscum and Stowe-Evans, 2000). However, phyA may be able to enhance phototropic curvature stimulated by UV-B just as it does the response to low fluence rate blue light (Stowe-Evans et al., 2001).

As shown in Figure 5.2, UV-A / blue light stimulates phototropism very effectively and so most research to date has focused on these wavelengths. One early hypothesis was that the cryptochromes, cry1 and cry2, could be primary photoreceptors in the UV-A / blue stimulated phototropic hypocotyl response (Ahmad et al., 1998b). Although this view has since been discredited (Lasceve et al., 1999, Stowe-Evans et al., 2001), we wanted to investigate whether the UV-B induced curvature seen in the present study could be elicited by the cryptochromes. Accordingly, the *phot1cry1cry2* triple mutant (which crucially already lacks the phot1 photoreceptor) was examined after illumination with 'UV-B Plus' and 'UV-B Minus' treatments (Figure 5.5) and the UV-B specific response found to be intact. It can therefore be concluded that the UV-B phototropic response presently under investigation is not dependant on either the cry1 or cry2 photoreceptor.

Because the *phot1-5phot2-1* double mutant was to form the basis of the forthcoming screen, neither of the known phototropin photoreceptors could interfere with the work nor be isolated in the screen. As a result of these preliminary studies, there was good reason to expect that the screen had the power to isolate novel mutants.

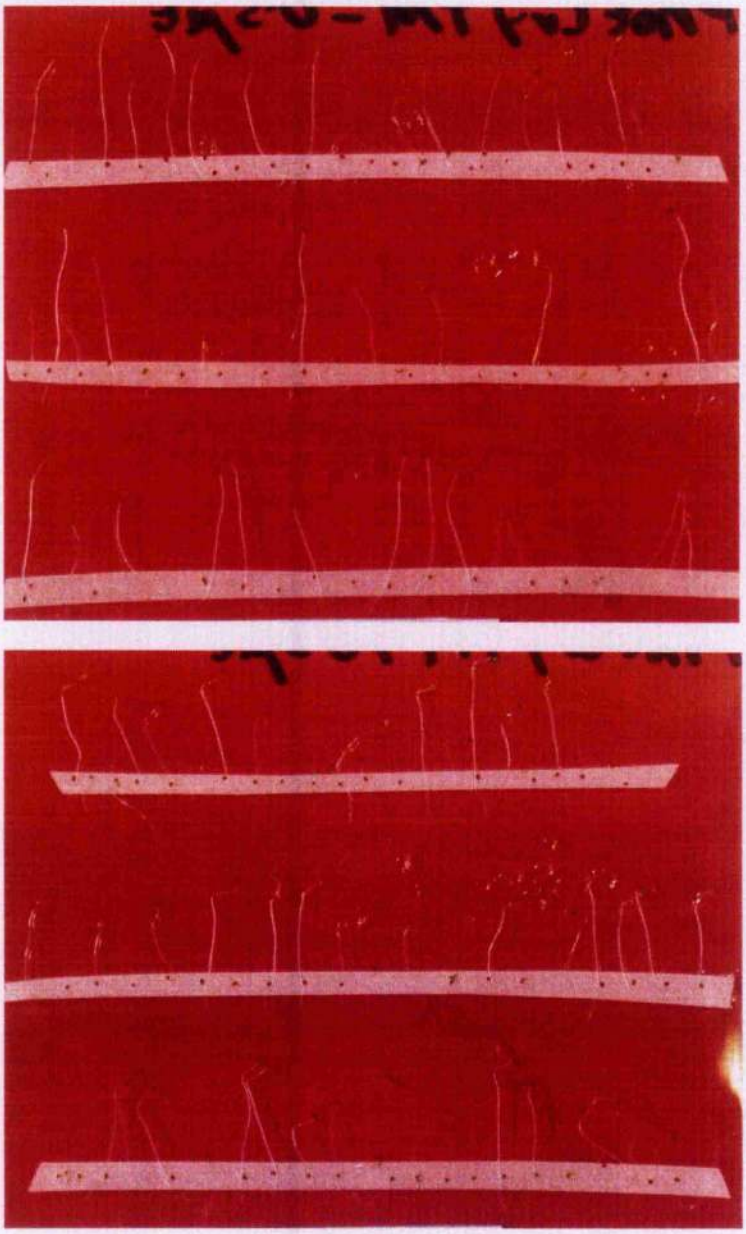


Figure 5.5
The UV-B specific phototropic hypocotyl response occurs in the *phot1cry1cry2* triple mutant implying that neither cryptochrome is the principal photoreceptor involved. Etiolated *Arabidopsis phot1-5cry1cry2* triple mutant seedlings (grown in darkness for just over 72 hours) were illuminated from the right hand side and for 72 hours with approximately 0.5 $\mu Em^{-2}s^{-1}$ UV-B (lower panel) or with a similar light treatment which lacked the UV-B wavelengths (upper panel). Directed hypocotyl curvature is clearly visible in the UV-B treated seedlings (lower panel) only. The seedlings were grown on media lacking sucrose.

5.6 Optimal Conditions for the Screen were Chosen After Altering a Number of Variables

Progress towards initiating a screen for mutants deficient in UV-B specific phototropism was hampered whilst the precise conditions under which to screen were optimized. UV-B induced hypocotyl curvature appeared to be very sensitive to particular treatment conditions and achieving consistency in the response was difficult. The addition of 2% sucrose to the agar plates together with the use of younger seedlings than those initially tested (around 60 hour-old seedlings in preference to ones over 70 hours old) appeared to improve the response. Preliminary experiments also suggested that 48 hour illuminations with 0.5 $\mu Em^{-2}s^{-1}$ UV-B as measured using a Skye Spectrosense UV-meter (equivalent to 0.7 $\mu Em^{-2}s^{-1}$ measured using a Macam spectroradiometer) could generate unambiguous curvature.

Testing the conditions chosen using the *phot1-5phot2-1* double mutant background with 'UV-B Plus' and 'UV-B Minus' illuminations (five replicates of each) suggested that a screen was indeed practical (Figure 5.6). Nearly all of the seedlings on the 'UV-B Plus' plates turned strongly towards the light source, whilst curvature shown by seedlings on the 'UV-B Minus' plates did not show the same consistent positive phototropism.

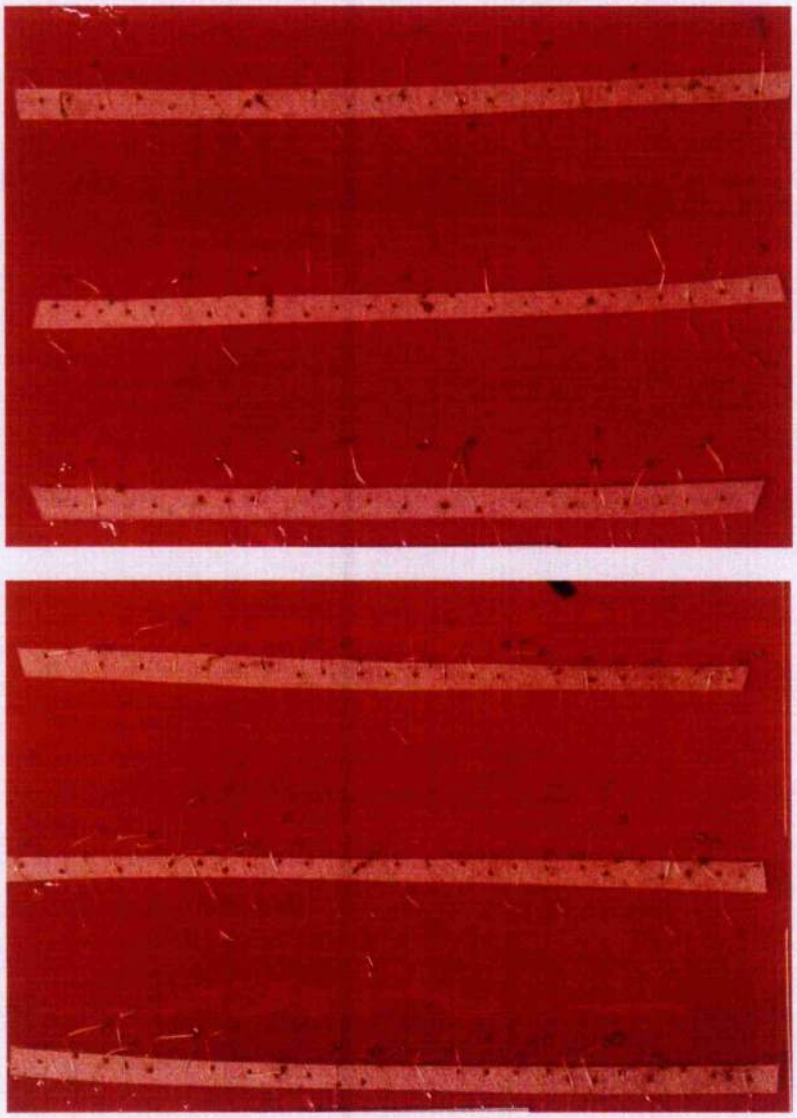


Figure 5.6
A screen for mutants deficient in UV-B induced positive phototropic hypocotyl curvature may be practical using carefully defined treatment conditions. Etiolated *Arabidopsis phot1-5phot2-1* double mutant seedlings (grown in darkness for 60 hours) were illuminated from the right hand side and for 48 hours with 0.5 $\mu Em^{-2}s^{-1}$ UV-B (lower panel), as measured using a Skye Spectrosense UV-meter, or with a similar light treatment which lacked the UV-B wavelengths (upper panel). Directed curvature is clearly visible in the UV-B treated seedlings (lower panel) only. The result shown was replicated four times (ie. the two plates displayed are representative of eight others). These seedlings were grown on media containing sucrose.

5.7 The Screen Produced Twenty-three Putative Mutants Apparently Deficient in UV-B Induced Positive Phototropic Hypocotyl Curvature

Mutageneses were performed on approximately 102,000 *phot1-5phot2-1* (Columbia ecotype) double mutant seeds in total using 0.6% Ethyl Methane Sulfonate (EMS). Approximately 6,920 M_1 plants were produced and allowed to self-pollinate and the resulting M_2 seed was separated into 173 batches, each batch containing seed from about 40 M_1 plants. Approximately 48,500 *phot1-5phot2-1* M_2 seedlings in total were screened, so about 7.0 M_2 seedlings were screened per M_1 plant. As described in Chapter Four, the recessive character of many mutant alleles dictates that a minimum of 4 M_2 individuals should be screened for each M_1 plant and so a value of 7.0 suggests that the screen will probably be successful in identifying most of the mutants which have been generated. The screen involved 60 hour-old etiolated seedlings being treated with 0.5 $\mu Em^{-2}s^{-1}$ UV-B for 48 hours before an assessment of the presence or absence of positive phototropic hypocotyl curvature was made. The mutagenesis and screening procedures are described in detail in the Materials and Methods section (Chapter Two of this volume).

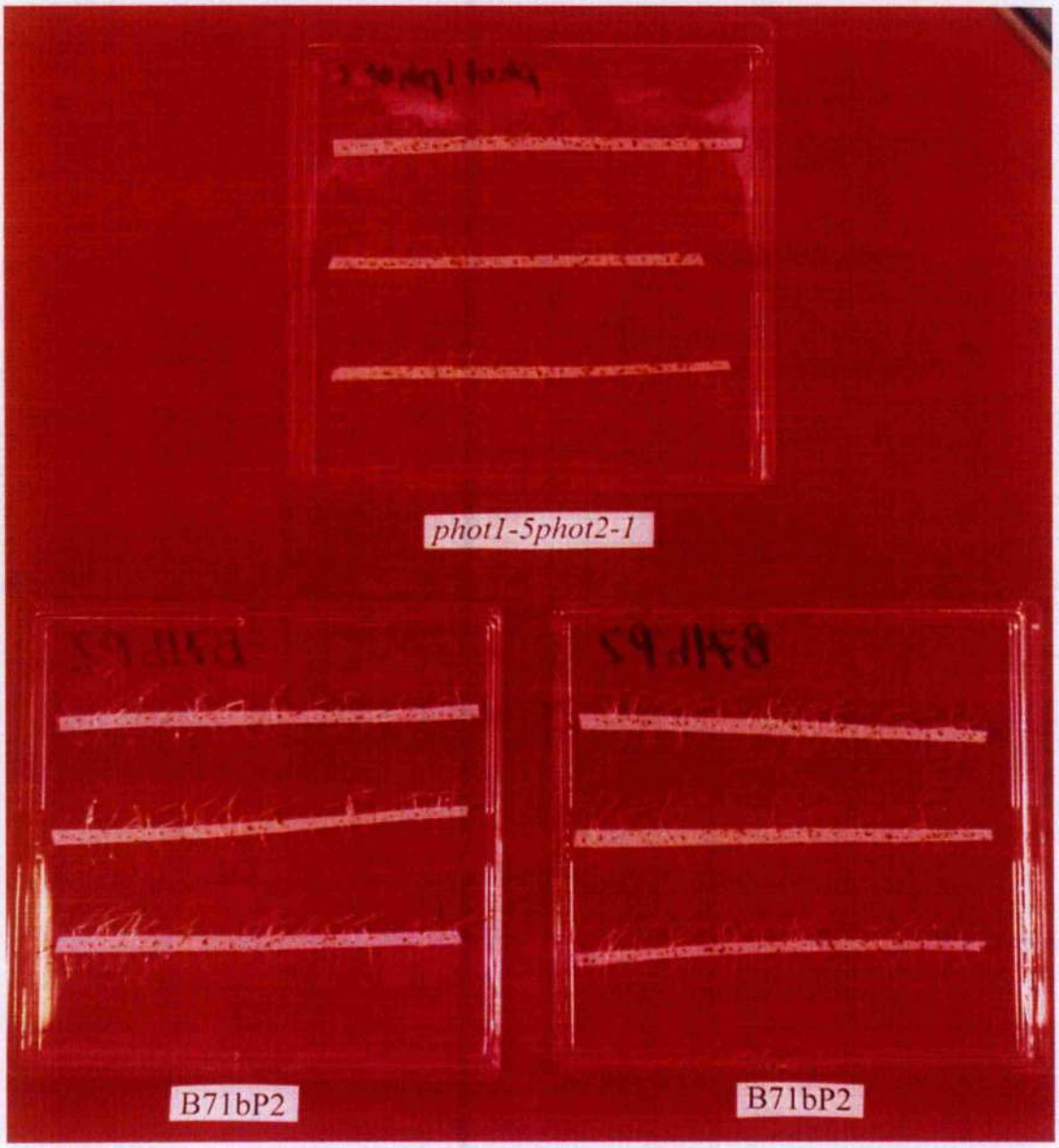
During the first stage of the screen 296 putative mutants, which appeared healthy but showed little or no curvature, were successfully isolated for closer examination. A large number of M_3 seedlings from each M_2 putative mutant was then sown on a single plate adjacent to a plate of *phot1phot2* control seedlings and the screening procedure repeated to try to identify putative mutants which were consistently deficient in UV-B induced phototropic hypocotyl curvature.

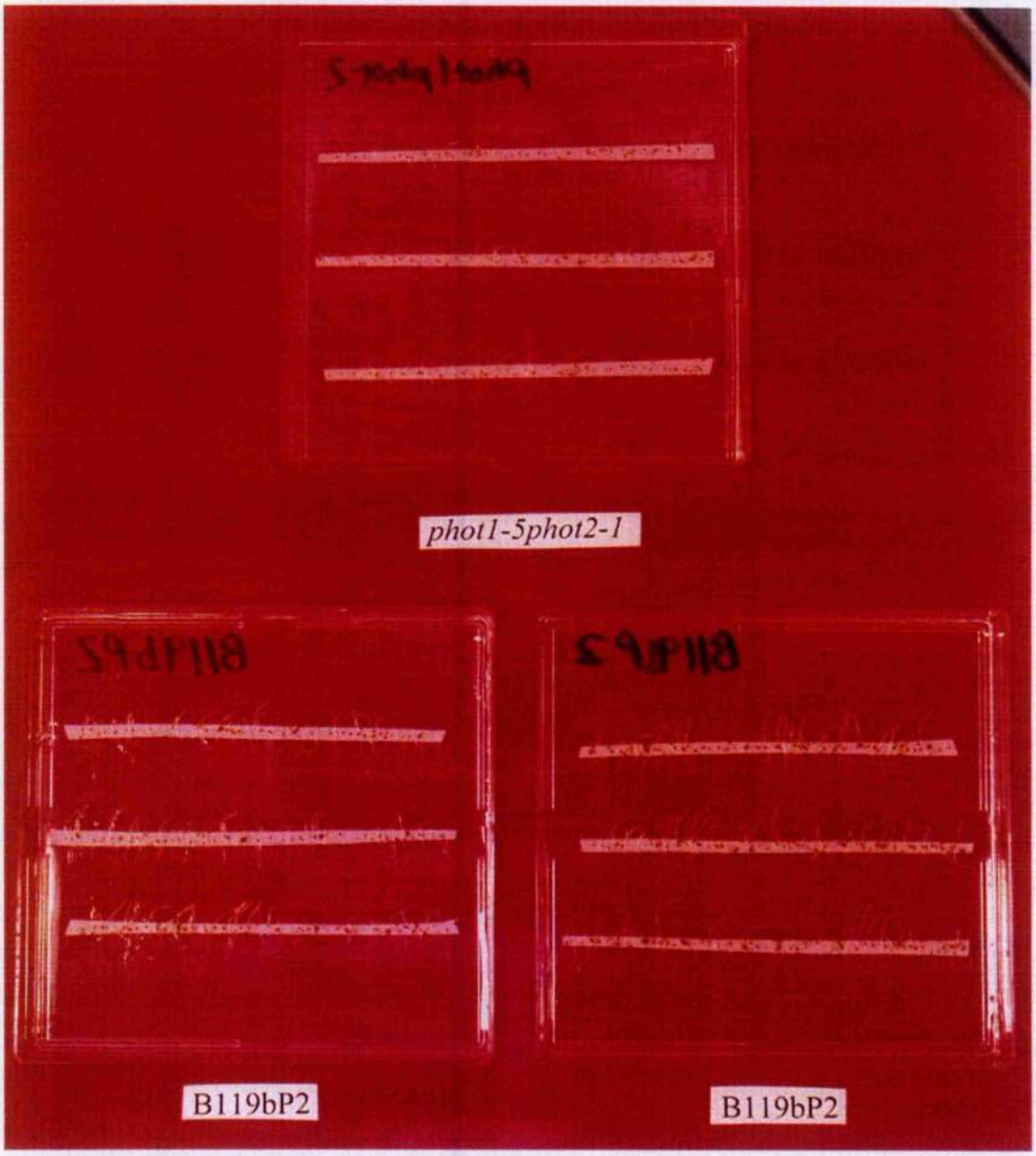
Of the 296 putative mutants examined, a mere 23 still appeared to lack a normal phototropic response to 48 hours of 0.5 $\mu Em^{-2}s^{-1}$ UV-B after being tested in quantity and these were set aside once more for a final and rigorous test to establish which, if any were true mutants.

5.8 Twenty-three Putative Mutants were Carefully Examined in the Final Analysis

The final stage of the screen was to check each of the twenty-three putative mutants, carefully sown and treated in proximity to the *phot1-5phot2-1* parent control line. Initially, of the twenty-three putative mutants twelve could be discarded as non-mutant, nine were equivocal (and were checked again) and two apparently retained a mutant phenotype and were re-examined on two plates each (see Figure 5.7). As part of the final study, a number of other photoreceptor mutants (*phot1-5phot2-1*, *phot1cry1cry2*, *phot1-5*, *phot2-1*, *cry1cry2hy1* and *phyAphyB*) were examined simultaneously under the screening conditions used to isolate the putative mutants and each was found to have a strongly positive phototropic response. Unfortunately, neither of the two putative mutants consistently exhibited a lack of UV-B induced phototropic hypocotyl curvature. Thus, no true mutants were identified as final products of this screen.

Figure 5.7
Each of the two selected putative mutants (B71bP2 and B119bP2) was again tested carefully using the screening conditions described against the parent *phot1-5phot2-1* line (shown at the top of each panel). Etiolated *Arabidopsis* seedlings (grown in darkness for 60 hours) were illuminated from the right hand side and for 48 hours with 0.5 $\mu Em^{-2}s^{-1}$ UV-B as measured using a Skye Spectrosense UV-meter. Unfortunately, neither putative mutant appeared to be a true mutant deficient in UV-B induced positive phototropic hypocotyl curvature as in each case, both replicates showed some directional hypocotyl bending.




phot1-5phot2-1
B119bP2
B119bP2

5.9 Discussion

The data described in this Chapter strongly indicates that *Arabidopsis* has a positive phototropic hypocotyl curvature response to UV-B wavelengths which occurs independently of either of the known phototropin photoreceptors. A series of pilot experiments were performed to optimize conditions for a screen which, in principle, had the power to isolate novel mutants of UV-B induced phototropism. The use of young seedlings and addition of sucrose to the growth media were found to enhance the phototropic response to UV-B, each perhaps for the same reason. UV-B wavelengths have little photosynthetic value to the plant and young seedlings with a supplemented energy source are likely to be more capable of responding than are older seedlings which have exhausted their limited energy reserves. Screening commenced after treatment conditions were produced which saw only a low percentage of *phot1-5phot2-1* (background genotype) seedlings fail to respond to UV-B and false positive putative mutants could therefore be reduced to a tolerable number.

5.9.1 There May be a Cumulative Explanation for the Discrepancy Observed Between the Number of Seeds Mutagenized and the Number of M_1 Plants Represented in the Screen

Approximately 102,000 *phot1-5phot2-1* seeds were mutagenized for use in the screen, described here, for mutants deficient in UV-B induced positive phototropic hypocotyl curvature. However, M_2 seed drawn from only about 6,920 M_1 plants were apparently represented in the screen itself. There is clearly a significant discrepancy between the two figures and the reason for this is not immediately clear. Nevertheless, since a similar discrepancy was found between the seeds apparently mutagenized and the M_1 plants represented in the UV-B induction of *CHS* mutant screen, described in Chapter Four, the cause of the inequality is worth brief consideration.

One possible reason why the two figures are different is that the method used to obtain each was different. The number of seeds mutagenized (102,000) was measured by weighing the seeds used in each mutagenesis, whereas the number of M_1 plants represented in the

screen came from multiplying the number of batches of M_2 seed prepared by the number of M_1 plants thought to have been harvested to generate each batch (ie. 173 batches x 40 M_1s). The different methods of measurement may introduce a significant amount of error. Each method will have its limitations. On the one hand, weighing may not be the most accurate method for estimating the number of seeds present in an eppendorf, whilst it could also be that in many cases seed was collected from more than the estimated forty M_1 plants for each batch.

Further possible explanations for the variation include the fact that a significant number of seeds will certainly be lost during the washes performed in the mutagenesis procedure and it is possible that a similar number may fail to germinate as a direct result of undergoing mutagenesis. Good evidence that increasing the concentration of EMS mutagen decreases the yield of viable plants was indeed obtained during the work described in Chapter Four (data not shown). Competition for light and space in a densely packed tray may also have played a role in drastically limiting the number of M_1 plants which actually set seed.

5.9.2 Problems Encountered During the Development of the Screen Suggested that it May be Difficult to Isolate Mutants Deficient in UV-B Induced Positive Phototropic Hypocotyl Curvature

Perhaps the biggest difficulty encountered during the present work was the fact that separating plant responses to UV-B from those to other wavelengths of light ideally requires that UV-B itself can be easily separated from other wavelengths. However, of the many hundreds of commercially available light filters, we were unable to identify one which transmitted only UV-B wavelengths and the use of monochromatic filters proved to be impractical. As a result, it was necessary to carry out studies, on *Arabidopsis* responses, using UV-B fluorescent tubes which emitted wavelengths of light other than UV-B and to compare these with further studies employing a filter which removed UV-B only (the 'Clear 130' filter).

One particular problem arose because it was considered important to ensure that there was more UV-A / blue light in 'UV-B Minus' illuminations than there was in 'UV-B Plus' treatments – to control for the possibility that responses believed to be stimulated by UV-B were in fact being elicited by longer wavelengths of light. This situation had added complications: for example the 'Clear 130' filter appears to reduce the transmission of UV-A light as it ages over several days. Another problem was that the 'Clear 130' filter doesn't actually eradicate UV-B completely; instead it reduces the UV-B fluence rate by about 90% (eg. 0.5 to 0.05 $\mu Em^{-2}s^{-1}$). Unfortunately, when studying a response, such as UV-B induced phototropism, which is sensitive to very low fluence rates of light, difficulties of this nature may be hard to overcome.

5.9.3 Problems Encountered Whilst Carrying Out the Screen Might Lead to the Suggestion of Improvements

In order to keep the number of false positives selected during the present screen to a minimum it was decided to select, as putative mutants, only seedlings which showed little or no curvature in either direction in response to UV-B treatment. This seemed a reasonable precaution because etiolated seedlings grow vertically, without any curvature in the absence of light. However, many of the *phot1-5phot2-1* double mutant seedlings treated with 'UV-B Minus' during the development of the screen showed a tendency to bend, although not towards the light. Perhaps then, it was a mistake to limit our selection of putative mutants to seedlings showing no curvature at all. Recent findings have suggested that 'random hypocotyl-bending' is produced by the action of the cryptochromes, cry1 and cry2 (Ohgishi et al., 2004). Retrospectively therefore, the *phot1cry1cry2* triple mutant might have formed a better basis for our UV-B non phototropic hypocotyl mutant screen, since random hypocotyl bending (clearly still present in the *phot1phot2* double mutant) should be very limited in the *phot1cry1cry2* triple mutant.

In conclusion, despite the failure of this particular screen to isolate mutants deficient in UV-B induced positive phototropic hypocotyl curvature, a response which can be elicited by UV-

B (and perhaps other wavelengths) does occur. It also is certain that phot1 and phot2 are not the only photoreceptors involved in phototropism and it seems likely that more than one additional photoreceptor exists. Functional redundancy provides a good explanation for why this screen failed to isolate any mutants.

CHAPTER 6

MUTANT CHARACTERIZATION AND IDENTIFICATION OF THE *30E5* GENE

6.1 Introduction

The *Arabidopsis 30e5* mutant was isolated during a transgene expression screen as a mutant showing reduced levels of *CHS-GUS* (chalcone synthase promoter fused to a β-glucuronidase reporter gene) activity when plants were exposed to UV-B light. Preliminary work, centring on northern blots, suggested that the *30e5* mutant may accordingly be impaired in light induced transcription of the endogenous *CHS* gene. The mutant, which has been backcrossed twice (*30e5bc2*) to the wild-type L. *er* background line, typically has green cotyledons but forms yellow / pale sectors on the true leaves. The pale sectors of the mutant leaf, which become more widespread under high fluence rate white light, were found to be low in chlorophyll and to, typically, contain developmentally arrested chloroplasts. In addition to showing a reduction in *CHS* transcript level, initial characterization also suggested that the *30e5* mutant may be impaired in *DFR* (dihydroflavonol reductase), *CHI* (chalcone isomerase) and *CAB* (LHCB1 chlorophyll a/b binding protein) gene expression. Significantly, anthocyanin accumulation in the mutant was found to be very much reduced (Fuglevand, Bilsland and Jenkins, unpublished work).

In the present work, studies were undertaken to further investigate aspects of the *30e5* mutant phenotype identified by the preliminary characterization. The focus was on altered responses to UV-B light treatment, although reductions in light induced *CHS* reporter gene activities were not found to be mirrored in the mutant's endogenous *CHS* gene expression. Significantly however, the *30e5* mutant proved to be far more susceptible to supplementary UV-B induced growth inhibition and leaf damage than is *uvr8*. Mutants with a similar visible phenotype to *30e5* were examined closely to see if any alleles of the mutant gene could be recognized and a labour intensive program of work was initiated to locate the *30E5* gene

which had already been mapped to the lower arm of *Arabidopsis* chromosome IV. As a result of fine mapping, the *30E5* gene was delimited to a small region of chromosome IV containing only 12-14 genes and a likely candidate identified.

6.2 *30e5* is an *Arabidopsis* Mutant Which Shows a Reticulate Leaf Phenotype and Accentuated Chlorosis Under High Fluence Rate White Light

The most striking aspect of the *Arabidopsis 30e5* mutant is its visible phenotype. Whilst *30e5* cotyledons are typically green, true leaves form yellow or pale sectors which give the plant a variegated appearance. Furthermore, a green reticulate pattern highlighting the vascular system can often be observed on mutant leaves (Figure 6.1).

Figure 6.1
A reticulate pattern highlights the vascular system of the *30e5* mutant leaf (right) compared to a uniformly green wild-type leaf (left). Each leaf was taken from a plant which had been grown for 17 days under continuous white light (25 $\mu Em^{-2}s^{-1}$).

When grown under high fluence rates of white light *30e5* is very much smaller than wild-type plants and there is a marked increase in the chlorosis of mutant leaves (Figure 6.2). Previous work had established that the chlorotic leaf sections of *30e5* are characterized by a reduction in chlorophyll content and abnormally developed chloroplasts (Bilsland, Fuglevand and

Jenkins, unpublished work). Unlike some other variegated mutants, such as *th-3* (Koornneef and Hanhart, 1981), restoration of the wild-type phenotype to *30e5* was not observed in response to the addition of thiamine.

Confirmation that the *30e5* lesion behaved as a single gene recessive mutation was provided by the 3:1 segregation of wild-type to mutant phenotype F_2 plants used in selecting a mapping population together with the unambiguously wild-type phenotype displayed in the F_1 progeny of *30e5* mutants backcrossed to wild-type L. *er* plants.

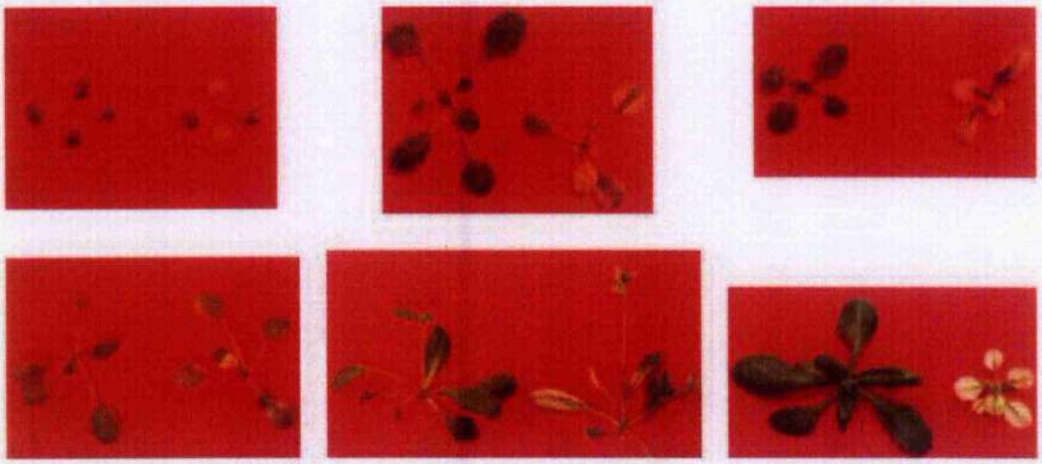


Figure 6.2
Growth under high fluence rates of white light results in a decrease in size and an increase in chlorosis of the leaves of *30e5bc2* mutant (on the right of each panel) compared to wild-type (on the left of each panel) plants. Plants were germinated and grown under 30 $\mu Em^{-2}s^{-1}$ (left-hand side panels), 100 $\mu Em^{-2}s^{-1}$ (centre panels) or 200 $\mu Em^{-2}s^{-1}$ (right-hand side panels) continuous white light respectively. Top row of panels – 14 day-old plants; Bottom row of panels – 21 day-old plants.

6.3 Although the *30e5* Mutant was Isolated as Having Low *CHS* Promoter Activity in Response to UV-B Treatment, Endogenous *CHS* Expression Studies Failed to Show a Consistent Reduction in Steady-State *CHS* Transcript Levels in Response to Treatment with Either UV-B or UV-A

The *30e5* mutant was isolated as having a reduced level of *CHS-GUS* expression in response to UV-B and other light qualities (Fuglevand and Jenkins, unpublished work). Confirmation

that *CHS* promoter driven expression of the *GUS* (β-glucuronidase) reporter gene, stimulated by different light qualities, is reduced in the mutant was obtained histochemically using a substrate of the GUS enzyme which gives rise to a blue product *in planta* (Figure 6.3).

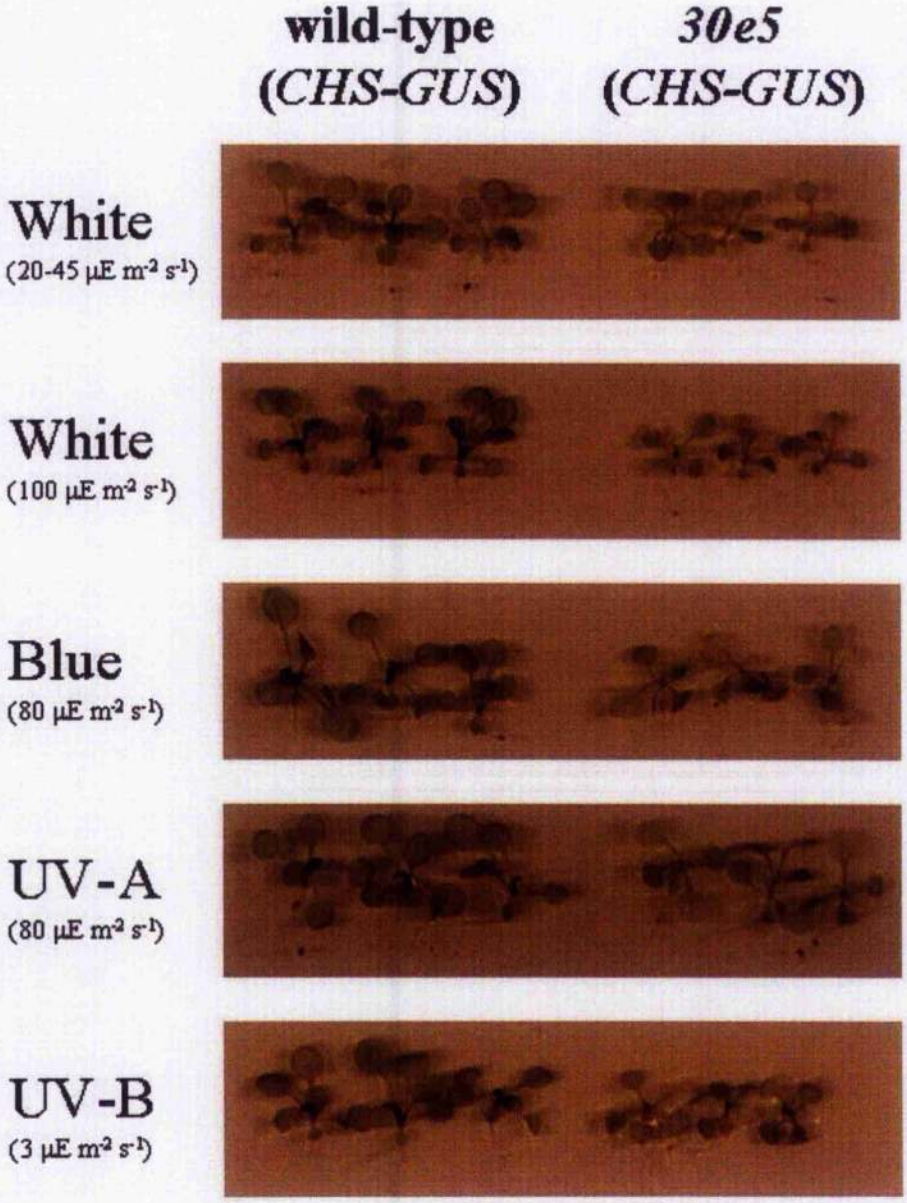


Figure 6.3
The level of *CHS-GUS* expression in response to various light treatments (which are described on the left) is reduced in the *30e5* mutant, compared to wild-type, as shown by the quantity of blue enzyme reaction product visible in each seedling. Seedlings were grown on compost under non-inducing conditions (LW) for 3 weeks before the illuminations shown were implemented for 6 hours (or 4 hours for UV-B). Finally, application of the enzyme substrate X-Gluc which results in the seedlings becoming stained with reaction product was used to highlight *CHS* promoter activity (see Materials and Methods).

To examine whether the reduced transgene expression was related to the choice of reporter gene, the *CHS* promoter - reporter gene studies on the mutant were extended by introducing a luciferase reporter transgene (*CHS-Luc*) into the *30e5bc2* line. The introduction of the transgene was accomplished by crossing *30e5bc2* to the *CHS-Luc 3.4* line and selecting a homozygous, *Luc* expressing, *30e5* phenotype individual from the resulting segregating population. *CHS* promoter driven expression of the *Luc* reporter gene, stimulated by different light qualities, was also found to be slightly reduced in the *30e5* mutant. Luminescent images of representative wild-type and mutant seedlings depicting *CHS* promoter – *Luc* reporter expression levels in response to different light treatments are shown below (Figure 6.4).

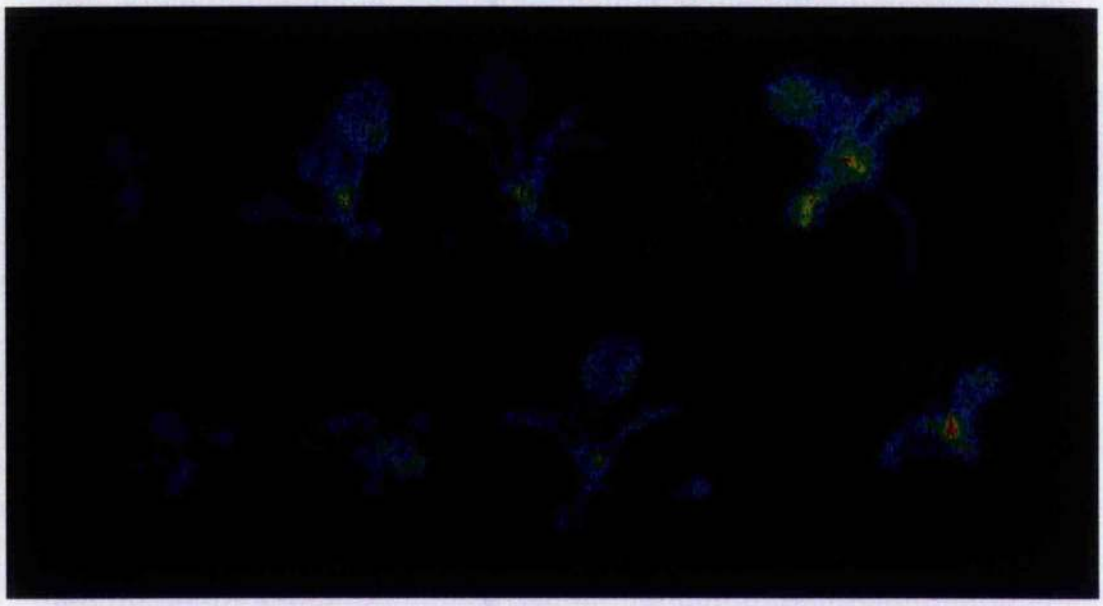


Figure 6.4
The level of *CHS-Luc* expression in response to various light treatments (left to right: low white light; 100 $\mu Em^{-2}s^{-1}$ high white; 80 $\mu Em^{-2}s^{-1}$ blue; 3 $\mu Em^{-2}s^{-1}$ UV-B) is slightly reduced in *30e5* mutant (bottom row) compared to wild-type (top row) seedlings, as shown by the generally diminished luminescence from mutant plants. Seedlings were grown on compost under non-inducing conditions (low white light) for 3 weeks before the light treatments described were provided for 6 hours (or 4 hours for UV-B) and *CHS-Luc* expression levels quantified (data not shown) using a Photek photon counting camera (see Materials and Methods).

Although reporter gene studies have shown that *30e5* has reduced *CHS* promoter activity in response to stimulating light treatments (Figure 6.3 and Figure 6.4), parallel work focusing on endogenous *CHS* gene expression (ie. repeated northern blots and RT-PCR) demonstrated that there is little or no reduction in steady-state *CHS* transcript levels induced by UV-A or UV-B treatment in the mutant (Figure 6.5).

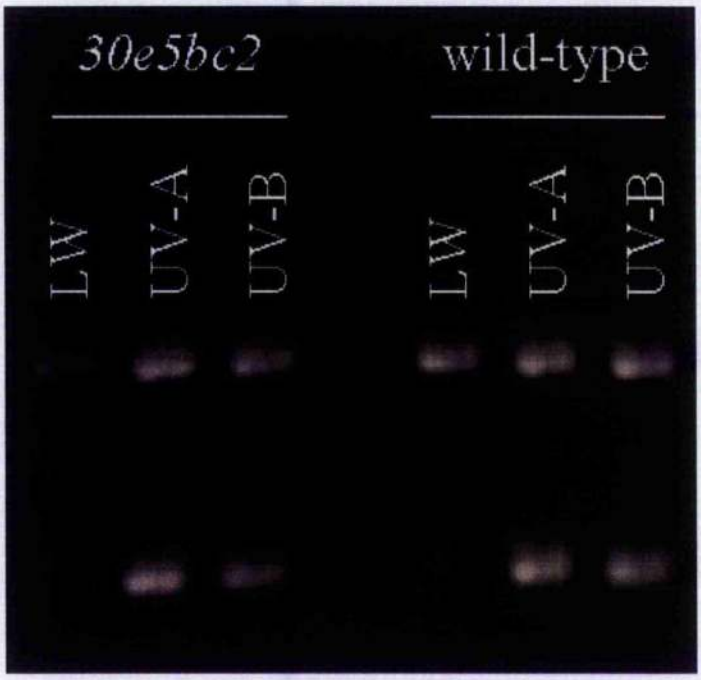


Figure 6.5
CHS steady-state transcript level (lower bands) is unaltered in the *30e5* mutant, compared to wild-type, in response to either UV-A (100 $\mu Em^{-2}s^{-1}$ for 6 hours) or UV-B (3 $\mu Em^{-2}s^{-1}$ for 4 hours) light treatment. The upper bands correspond to *ACTIN* gene expression and represent a loading control. Seedlings were grown on compost under non-inducing low white light for 3 weeks before the light treatments described were provided. RNA was extracted from leaf tissue and *CHS* gene expression quantified by reverse transcriptase PCR (see Materials and Methods).

If chalcone synthase mRNA accumulates normally in the leaf epidermis of our mutant, then the similarities between *30e5* and *cue1* (Li et al., 1995) are too striking to ignore. Both mutants have a reticulate leaf phenotype (reflecting normal bundle sheath cells) and a variegated appearance which becomes increasingly chlorotic during growth under high fluence rates. Both *30e5* and *cue1* have aberrant chloroplast structure in the chlorotic sections of the leaf and reportedly manifest deficiencies in *CAB* gene expression and anthocyanin accumulation. In addition, *cue1* displays abnormalities (reductions in cell number and

alterations in cell shape) of the palisade mesophyll cell layer. Notwithstanding these observations, *30e5* is not an allele of the *CUE1* gene. *CUE1* maps to chromosome V (Streatfield et al., 1999) as described by The *Arabidopsis* Information Resource (http://www.arabidopsis.org/servlets/TairObject?id=27234&type=gene) and encodes the plastid inner envelope phosphoenolpyruvate / phosphate translocator (PPT) (Li et al., 1995, Rodermel, 2002, Voll et al., 2003), whereas the *30E5* gene is located on *Arabidopsis* chromosome IV (see Section 6.6). The significance of the similarities between the *30e5* and *cue1* mutants is not altogether clear but it is possible that each phenotype stems from deficiencies in chloroplast development.

6.4 The *30e5* Mutant is Hypersensitive to Supplementary UV-B Induced Growth Inhibition and Leaf Damage

One of the most interesting characteristics of the *30e5* mutant is the increase in chlorosis which occurs during growth under progressively higher white light fluence rates (Figure 6.2). A possible explanation for this phenomenon is that *30e5* is more susceptible to oxidative stress than are wild-type plants. Preliminary experiments investigating a possible role for oxidative stress in producing the increase in chlorosis showed that an 8 hour, unsupplemented UV-B (3.0 $\mu Em^{-2}s^{-1}$) treatment may indeed increase the extent of chlorosis in approximately 3 week-old, low white light grown *30e5* plants after a suitable recovery period of about 3 days. However, spraying on various concentrations of exogenous hydrogen peroxide (H_2O_2), a common source of endogenous oxidative stress, failed to have a similar effect on the mutant.

Previous work on a backcrossed mutant line (*30e5bn2*) had shown that *30e5* was afflicted with a significant reduction in capacity for DNA protection. Sixteen hour treatments of up to 6.0 $\mu Em^{-2}s^{-1}$ UV-B produced an increase in the level of measurable DNA damage (both 6-4 photoproducts and thymine dimers) found in the *30e5* mutant compared to a wild-type control (Taylor, Fuglevand and Jenkins, unpublished work).

As a result of the findings described above, it was thought likely that the *30e5* mutant would have an increased susceptibility to supplementary UV-B induced growth impairment and leaf damage and this was indeed demonstrated (Figure 6.6) using the experimental conditions developed in Chapter Three.

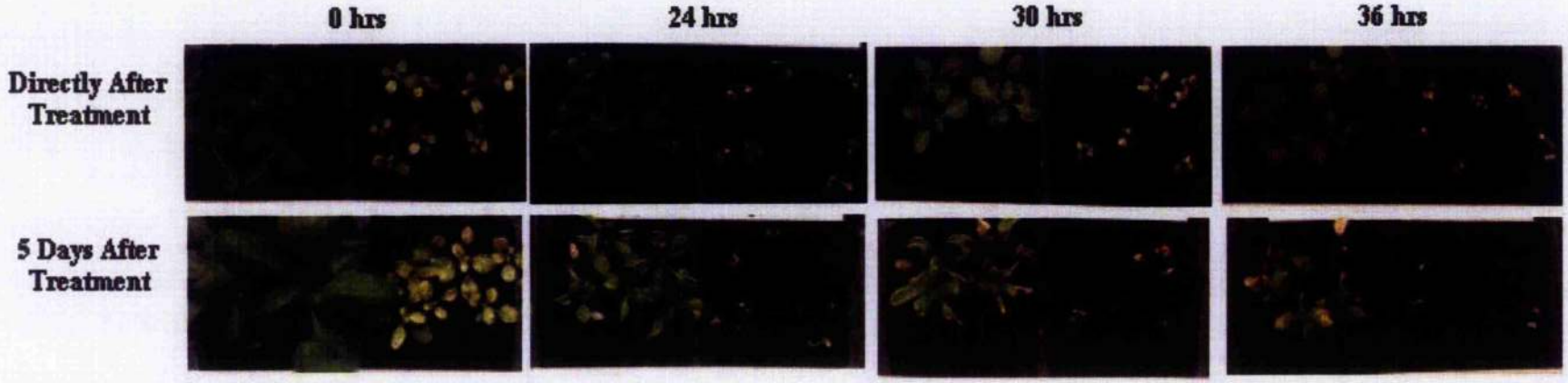


Figure 6.6
The *30e5* mutant is extremely sensitive to supplementary UV-B induced growth impairment and leaf damage. Wild-type L. *er* (left of each panel) and *30e5bc2* mutant (right of each panel) were grown under 120 $\mu Em^{-2}s^{-1}$ white light for 12 days before treatments of varying durations (as indicated) with 5 $\mu Em^{-2}s^{-1}$ UV-B (Supplemented with 40 $\mu Em^{-2}s^{-1}$ white light) and then returned to 120 $\mu Em^{-2}s^{-1}$ white light for a 5 day recovery period.

The *cue1* mutant has not been tested to determine its level of susceptibility to UV-B induced DNA or leaf tissue damage but other aspects of the phenotype strongly suggest that, like *30e5*, *cue1* would show an increase in vulnerability to UV-B just as it does to high intensity white light. The *cue1* mutant lacks the phosphoenolpyruvate / phosphate translocator (PPT) found on the inner plastid envelope, which imports phosphoenolpyruvate (PEP) to the chloroplast. The pre-chorismate part of the shikimate pathway is confined to plastids and requires PEP as its first substrate. Once synthesized, chorismate is used to make the aromatic amino acids including phenylalanine – and this can be further metabolized in phenylpropanoid biosynthesis. Phenylpropanoids in turn, have a variety of roles in plant secondary metabolism, including as UV protectants and antioxidants. Clearly a lack of PEP in the stroma of mutant chloroplasts is a key factor in producing the *cue1* phenotype. However, accumulating data suggests that *cue1* cannot simply be explained by a general deficiency of the shikimate pathway and it has recently been proposed that a shortage of signalling molecules, possibly derived from phenylpropanoid metabolism and acting as key developmental triggers, could be responsible for the *cue1* mutant phenotype (Streatfield et al., 1999, Voll et al., 2003, Knappe et al., 2003, Rodermel, 2002).

6.5 The *30e5* Mutant Gene is an Allele of the *DOV1* Gene

Before embarking on a lengthy map-based cloning project, we wanted to be confident that the *30e5* mutant gene had not already been located under a different name. To try to identify mutants deficient in genes which could be allelic to *30E5*, literature and seed stock searches were initiated using keywords such as 'reticulated', 'variegated' and 'chlorotic'. Mutations mapping to locations in the *Arabidopsis* genome other than the 30-35 cM region known to contain *30E5* (such as *var2*, *chm1*, *pac*, *reticulata*, *th2*, *th3*, *cue1* and *immutans*) were eliminated from consideration. Efforts were made to acquire seed from remaining mutants phenotypically similar to *30e5*.

Many mutant alleles (including *30e5*) are recessive. If two distinct mutants, deficient at separate locii with recessive alleles, are cross-fertilized the F_1 progeny will display a wild-type phenotype as the non-mutant alleles at each locus will complement the mutant alleles from each parent. Conversely, if two mutants are deficient in allelic genes then the F_1 progeny produced by cross-fertilization should display a mutant phenotype also. Cross-fertilizing particular mutants with a wild-type plant should indicate whether a corresponding mutant allele is recessive. In this way it was demonstrated that the *30e5* mutation lies in an allele of the *DOV1* (cf. NASC seed stock #N557) / *F196* (cf. NASC seed stock #N458) gene. Progeny resulting from cross-fertilization of *30e5* and *dov1* mutants can be seen below (Figure 6.7).

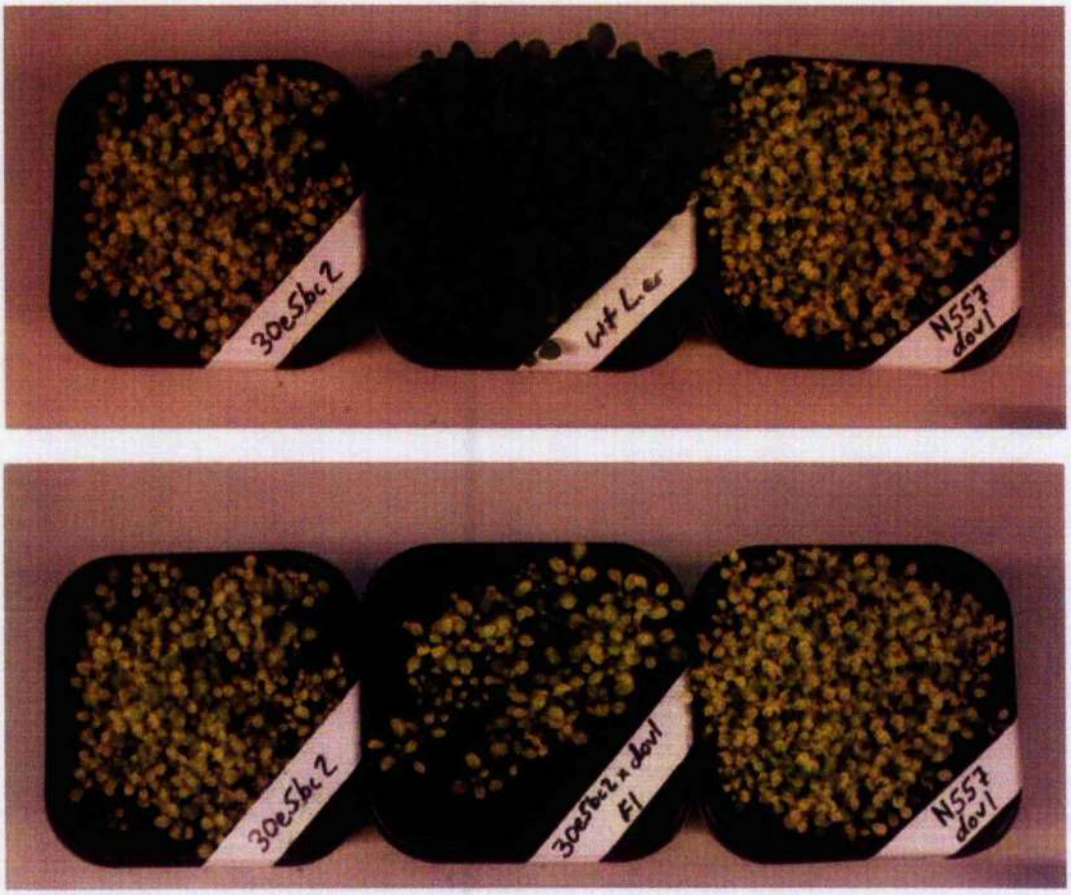


Figure 6.7
The *30e5* and *dov1* mutants harbour allelic mutations. The *30e5* mutant is shown on the left-hand side of each panel, the *dov1* mutant is on the right hand side of each panel. In the centre of the upper panel wild-type L. *er* is shown for comparison and in the centre of the lower panel the F_1 progeny of cross-fertilization between the *30e5* and *dov1* mutants clearly shows a mutant (rather than wild-type) phenotype.

Although initial characterization suggested that the *dov1* mutant takes a longer period of time to flower than does *30e5*, the lack of complementation (Figure 6.7) demonstrates that the two harbour mutations in the same gene. The *dov1* (differential development of vascular-associated cells) mutant was isolated during a screen for *Arabidopsis* reticulate leaf mutants which was initiated to identify genes playing a role in the differential development of bundle sheath and mesophyll cell chloroplasts (Kinsman and Pyke, 1998). The *dov1* mutant shows a cell-specific difference in chloroplast development. Mutant leaves are reticulate displaying the same pattern, which highlights the vascular system, present in *30e5*. The *dov1* bundle sheath cells always have normal chloroplasts but mesophyll cell chloroplasts are structurally abnormal and reduced in number. The *dov1* mutant also retains green cotyledons and segregation of the phenotype is indicative of a single gene recessive nuclear mutation (Kinsman and Pyke, 1998). Furthermore chlorosis in the *dov1* mutant may be accentuated under high light fluence rates. No attempt has been made to clone the *DOV1* gene (K. A. Pyke, personal communication).

6.6 Mapping the *30E5* Gene

6.6.1 Identification of Polymorphic Markers Close to the *30E5* Gene Using a Standard F_2 Mapping Population of 1000 DNA Samples

Previously, the *30e5* mutation had been mapped to a 30-35 cM interval on the lower arm of *Arabidopsis* chromosome IV, between the co-dominant cleaved amplified polymorphic sequence (CAPS) markers *PG11* and *DHS1*. In addition, F_2 seed which would form the basis of a mapping population had been generated by crossing *30e5* (L. *er* background) to wild-type (Col-3 background) and allowing the progeny to self-fertilize (Fuglevand and Jenkins, unpublished work). Next, a standard mapping population was created by extracting DNA from 1000 *30e5* phenotype F_2 individuals and a series of markers within the delimited region, many from the Cereon *Arabidopsis* Polymorphism Collection

(http://www.arabidopsis.org/cereon/), were tested to see which produced utilizable polymorphisms, distinguishing DNA from the L. *er* and Columbia genomes respectively (Jander et al., 2002, Bell and Ecker, 1994). Three polymorphic markers *Cer 460 656* (*T4L20-100*), *Cer 460 658* (*T4L20-113 / F11I11-3*) and *Cer 424 643* (*F11I11-30*), spanning two bacterial artificial chromosomes (T4L20 and F11I11), were found to be so close to the mutation that no recombination breakpoints could be found between these markers and *30E5* within the standard mapping population of 1000 F_2s. As a result, it can be calculated that the mutant gene is less than 0.05 cM from each of the three markers. If 1 cM is equivalent to, very approximately, 250 kb (Jander et al., 2002, Lukowitz et al., 2000), then each marker should be less than about 12.5 kb from *30E5* - although in fact the three markers span 40 kb. Further mapping uncovered a recombinant F_2 at *Cer 460 650* (*T4L20-89*). The marker *Cer 460 650* consequently becomes a flanking marker, delimiting the location of the *30E5* gene on the left-hand (upper) side (see Figure 6.8). Table 2.2 describes the polymorphic markers used to map the *30E5* gene.

6.6.2 Analyses of Candidate Gene Sequences from the Mutant did Not Identify the *30E5* Gene

The number of F_2 plants required to provide any recombinants in a given genetic interval increases dramatically as the size of the interval decreases. Once the genomic region of interest in a map-based cloning project has been delimited to less than 40 kb few further recombinants are likely to be found, even in a very large mapping population, and the whole region should be sequenced (Jander et al., 2002). Primers were therefore designed to amplify 42 (overlapping as necessary) fragments (each < 1 kb) corresponding to the 21 predicted genes between *T4L20-89* (upper flanking marker) and the location *F11I11-45*. Mutant phenotype F_2 DNA was chosen for sequencing and the sequencing results (provided by the Sir Henry Wellcome Functional Genomics Facility, University of Glasgow) were analyzed by BLAST searches against the L. *er* and Columbia genomes. Since the *30e5* mutant was generated by Ethyl Methane Sulfonate (EMS) mutagenesis it was noted that bias towards a C-

to-T or G-to-A change respectively (S. Naito, Hokkaido University, *Arabidopsis* Newsgroup correspondence, 2002) might be expected. However, the sequencing results were incomplete and highlighted too many changes rendering these unlikely to identify the *30e5* mutation. In retrospect, a high-fidelity polymerase (such as *Pfu* DNA polymerse) should have been used to generate the DNA fragments for sequencing as this would have reduced sequencing inaccuracies.

6.6.3 The Use of a Specially Selected Fine-Scale Mapping Population of 66 DNA Samples, Derived from Plants with Recombination Breakpoints Close to the Right Hand Side (RHS) of the *30E5* Gene, Identified a Lower Flanking Marker Close to the RHS of *30E5*

Increasing the size of the standard F_2 mapping population substantially would have been a labour intensive step, and one which offered no guarantee of additional recombinants adjacent to the mutant locus. Therefore, it was decided that a fine-scale mapping population, enhanced for recombinants in the region of interest, should be made to delimit the location of the *30E5* gene still further. Accordingly mutants generated in the Columbia background (since *30e5* is L. *er*) were sought using the NASC database to search for seed which would be readily available. It was very important that the locus chosen should be extremely close to *30E5* and that the corresponding mutant had a readily identifiable phenotype, in order to make the exercise both worthwhile and practical. The *FAH1* (ferulate-5-hydroxylase) locus resides on BAC F23E13, about 500 kb from the estimated location of *30E5*, and the recessive *fah1* mutant phenotype (Chapple et al., 1992) is a characteristic red fluorescence under UV-A (eg. 365 nm transilluminator). The *30e5* and *fah1* mutants were crossed together and the F_1 progeny allowed to self-fertilize to generate a very large quantity of F_2 seed. The F_2 plants were grown up, but only *30e5* phenotype F_2s were retained and seed was collected from each plant separately. A very small proportion of the *30e5* phenotype F_2 individuals will have a recombination breakpoint between the two key locii, but because *fah1* is also a recessive mutation F_3 plants must be examined in order to identify the recombinants. Examining the F_3

progeny from a large number of *30e5* phenotype F_2s separately showed that a few were segregating for the *fah1* mutant phenotype. One *30e5fah1* double mutant was retained for DNA extraction from each F_2 which gave rise to segregating double mutant F_3s. In this way, a fine-scale mapping population was generated consisting of 66 individual DNA samples each containing a recombination breakpoint between *30E5* and the neighbouring *FAH1* gene.

By analyzing the fine scale mapping population, four recombinants were detected at locus *Cer 424 643* and three of these were found to be recombinant also at *F11I11-22* (a polymorphism not found on the Cereon database but revealed after comparing our sequence data for 'Gene 15' with the Landsberg and Columbia genomic sequences). Accordingly, *F11I11-22* becomes the right-hand side (lower) flanking marker of *30E5*. All the recombinant DNA samples described were found to be non-recombinant at *Cer 460 658* and *Cer 460 656*, attesting to their reliability. Thus, *30E5* was delimited to around 40 kbp and 12-14 candidate genes. It should be noted that 'Gene 15' (see Figure 6.8) could not be excluded from consideration with absolute certainty (as the lower flanking marker *F11I11-22* occurs within its coding sequence) and also that a separate annotation of the genomic region of interest (found on the SIGnAL-SALK website) predicts the existence of an additional gene (At4g34695) not recognized previously. No further polymorphisms could be identified and the delimited location of the *30E5* gene is shown in Figure 6.8.

GENES

3. GATA transcription factor (At4g34680)
4. Hypothetical protein (At4g34690)
5. Putative protein (At4g34700)
6. Arginine decarboxylase (At4g34710)
7. Vacuolar H^+ transporting ATPase 16K chain (At4g34720)
8. Putative protein (At4g34730)
9. Amidophosphoribosyltransferase 2 precursor (At4g34740)
10. Putative protein (At4g34750)
11. Putative auxin regulated protein (At4g34760)
12. Putative protein (At4g34770)
13. Putative protein (At4g34780)
14. Putative protein (At4g34790)

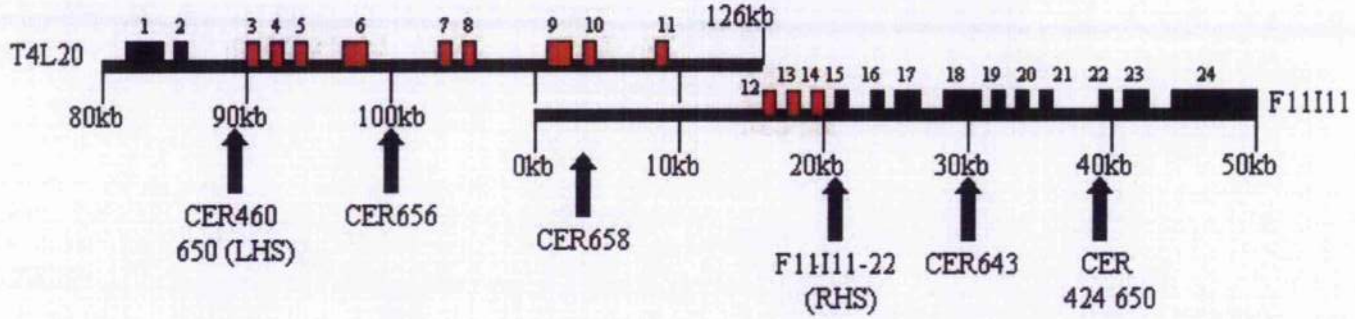


Figure 6.8
Location of the markers used to map *30E5* and the candidate genes. The two overlapping bacterial artificial chromosomes (T4L20 and F11I11) are shown together with the polymorphic markers used to map *30E5* (*Cer 460 650*, *Cer 460 656*, *Cer 460 658*, *Cer 424 643*, *Cer 424 650* and *F11I11-22*. The Flanking markers on either side of *30E5* (LHS and RHS) are indicated and the candidate genes between these markers are shown in red with the corresponding annotations given above. This map was constructed using information from TAIR (http://www.arabidopsis.org/).

6.6.4 Examining a Number of Tagged Lines for the Mutant Phenotype Did Not Identify the *30E5* Gene

In order to try to identify the *30E5* gene, several tagged lines from the SALK T-DNA collection were ordered. Unfortunately, none of the tagged lines obtained displayed, or were segregating for, a mutant phenotype (Figure 6.9). Studying the location of the putative T-DNA insertion sites for each of the twelve lines suggested that five candidate genes (Gene numbers 3, 5, 8, 9 and 13 on Figure 6.8) should have been inactivated, since these genes would be likely to contain insertions within exons. However, Southern blotting using the labelled gene as a probe was not employed to determine whether each tagged line really contained an insertion within the gene expected and so none of the candidate genes could be unequivocally ruled out.

Figure 6.9
None of the SALK T-DNA lines examined showed the *30e5* mutant phenotype. The three pots along the top row contain control plants; left to right: wild-type L. *er*, *30e5* and *dov1*. The latter two mutants display their characteristic chlorotic phenotype (ie. are generally yellow rather than green) unlike any of the twelve pots below containing the SALK T-DNA Lines (rows 2-5). Plants were grown in 140 $\mu Em^{-2}s^{-1}$ white light for 16 days.

6.6.5 Identification of the *ATase2* (At4g34740) Gene as a Candidate for *30E5*

Having delimited the location of the *30E5* gene to 12-14 candidate open reading frames (Figure 6.8), it was decided that a closer examination of each gene might lead to the identification of one which when disrupted could give rise to a chlorotic leaf phenotype in the plant. As part of these studies, it was noted that an *Arabidopsis* mutant called *atd2* which has white leaves and green cotyledons had been described (van der Graaff, 1997, Rodermel, 2002). The *ATD2* gene encodes a glutamine 5-phosphoribosylpyrophosphate amidotransferase (ATase2) which is one of three isoenzymes that catalyze the first step of *de novo* purine biosynthesis. One of our 12-14 *30E5 / DOV1* candidate genes is described as an "Amidophosphoribosyltransferase 2 precursor" and this has the same gene identification number (At4g34740) as *ATD2*. The *ATD2* gene was identified by T-DNA tagging some time ago (van der Graaff, 1997), but has recently been reported as cloned under two separate names: *atd2* or ATase2 deficient (van der Graaff et al., 2004) and *cia1* or chloroplast import apparatus deficient (Hung et al., 2004). The striking similarities which these mutants each show to *30e5* (eg. green cotyledons, chlorosis of the true leaves, retardation of growth and chloroplast abnormalities (van der Graaff et al., 2004, Hung et al., 2004)) together with the fact that the gene responsible for the *atd2 / cia1* mutations is one of only a few within the 40 kbp delimited by the map-based cloning data presented here, strongly suggests that *ATD2 / CIA1* (At4g34740) is also *30E5 / DOV1*. Surprisingly however, two of the T-DNA tagged lines previously examined (Figure 6.9) were thought to contain insertions in gene At4g34740 - although the presence of these insertions was never actually confirmed. Additionally, the *atd2* mutant phenotype is more extreme (the plants are smaller, more fragile and possibly more chlorotic) than that of *30e5* or *dov1*. Examining the F_1 progeny of a cross between the two plants should reveal whether or not the *30e5* mutant contains a novel allele of the *ATD2* gene since the *atd2* mutation is also recessive. This latter study is currently in progress.

6.7 Discussion

6.7.1 Differences Inherent in Examining Gene Expression Using a Reporter Gene Compared to Measuring Endogenous Transcript Levels May Explain Why *CHS* Expression in the *30e5* Mutant Appeared to be Reduced in Transgene But Not in Transcript Studies

The *30e5* mutant was initially chosen for study on the basis of its apparently lowered *CHS* expression levels, compared to wild-type, when illuminated with UV-B or other inductive light qualities. The belief that the *30e5* mutant had deficiencies in its capacity for light induced *CHS* gene expression was largely rooted in incipient studies using a *CHS-GUS* transgene. Support for the conclusion that the mutant shows a reduced level of light stimulated *CHS* promoter activity was found in the present work also using the *CHS-GUS* transgene and extended during scrutiny of the activity of a *CHS-LUC* transgene (see Section 6.3). However, studies on endogenous *CHS* gene expression have consistently failed to sustain the results obtained for the transgenes. Work involving northern blotting and RT-PCR (Figure 6.5) indicates that the quantity of steady-state *CHS* transcripts accumulating in the illuminated *30e5* mutant is not less than that produced in similarly treated wild-type plants.

Why the data from transgene and transcript studies respectively, on stimulation of the *CHS* gene promoter are discrepant is not immediately obvious. However, an explanation contingent on the use or introduction of one reporter gene or the other (*GUS* or *LUC*) would seem to be excluded by the similar results obtained in each case. One possible resolution of the paradox may emerge from the fact that transgene and transcript studies measure different things. Whilst the reporter gene studies are quantifying *CHS* promoter activity, the transcript studies (northerns and RT-PCR) are monitoring steady state mRNA levels. It is possible, for example, that whilst less *CHS* mRNA is synthesized in the *30e5* mutant in response to inductive illuminations, less too is being degraded – although there is little information on *CHS* transcript stability and the gene is principally regulated at the level of transcription.

More work may produce an answer to this question, but whether more work is worthwhile when the mutant shows no alteration in endogenous *CHS* transcript accumulation is doubtful.

6.7.2 Perhaps a Greater Emphasis on the use of *Arabidopsis* Mutant Information Resources at Different Stages of Map-Based Cloning would have Meant that Candidate 30E5-Encoding Genes could have been Identified Earlier

Experience is what you get when you didn't get what you wanted and it is often a lot easier to see the best decisions to have made long after it is too late to make them. The attempt to identify the *30E5* gene by a map-based cloning strategy was not badly conceived, nor was it badly executed or beset with problems. Nonetheless, it now seems likely that the *30E5* gene had been identified (as *ATD2*) before the present project had begun (van der Graaff, 1997). Despite the recent advances, both technically and in the quality of resources available (Lukowitz et al., 2000, Jander et al., 2002), map-based cloning is still a demanding and labour intensive activity and the likelihood that the gene responsible for a particular mutant phenotype has already been identified elsewhere increases with every passing year. Consequently, before progressing from stage to stage in a map-based cloning project an appropriate amount of time should be devoted to asking whether or not likely candidate genes can be identified from the information already gathered.

Initially, a description of the mutant phenotype alone should be used as a basis for searches of germplasm stocks (eg. the Nottingham and Ohio *Arabidopsis* Stock Centres) and also used in literature searches for published work on mutants with similar phenotypes. All the possible descriptive terms for a particular phenotype should be tried in the seed stock and literature searches since only exact word-for-word matches will find putative alleles of the mutant gene sought. The importance of using of a range of keywords for an exhaustive search is highlighted by the work on *30e5*. A search of the NASC germplasm database did not identify *atd2* because the keywords used in the searches (eg. varieg*, reticul*, chlorotic, bleach*) did not correspond to the *verbatim* description of the *atd2* phenotype ("white leaves with green veins").

Once an initial map position has been obtained for a mutant gene of interest it is sensible to examine the annotated genes in the vicinity to identify ones which if disrupted could give rise to the mutant phenotype. Many genes in the *Arabidopsis* genome are of unknown function but those for which a function has been identified may be associated with a mutant phenotype which can be compared with that of the mutant of interest. If potentially allelic mutations can be identified then the corresponding mutants might be tested for allelism as described in Section 6.5 for *dov1* and *30e5*. Unfortunately, none of the genes in the vicinity of *30E5* suggested to us that they could be responsible for a chlorotic / variegated phenotype until the *atd2* mutant was noticed in a review of variegated mutants (Rodermel, 2002). Perhaps the precise functions of all the candidate genes in the immediate vicinity of *30E5* should have been established and the related literature examined in more depth before proceeding to the fine-mapping stage of the project.

6.7.3 The *atd2 / cia1* Mutant Phenotype is the Result of a Deficiency in *de novo* Purine Biosynthesis

It is probable, though not certain, that the *30e5*, *dov1* and *f196* mutants which are defective in the same gene, contain novel alleles of *ATD2 / CIA1*. The *ATD2* gene (At4g34740) encodes ATase2, one of at least three isoenzymes catalyzing the first committed step of *de novo* purine biosynthesis in *Arabidopsis* (van der Graaff et al., 2004). Purine nucleotides are of critical importance in plant growth and development providing a basis for the biosynthesis of nucleic acids, B vitamins, polysaccharides, essential cofactors (eg. nicotinamide and flavin coenzymes) and plant hormones.

The *atd2* mutant, created in a C24 background (van der Graaff et al., 2004), and *cia1* which also contains a recessive mutant allele of the *Arabidopsis* ATase2 gene (Hung et al., 2004) have been characterized separately. The *cia1* mutant was isolated as a result of deficiencies in its ability to import proteins into the chloroplasts (Hung et al., 2004). Each mutant is reduced in size and was found to have normal green cotyledons, but pale coloured leaves which become more chlorotic under high fluence rate growth conditions. The chlorosis appears to

reflect abnormalities in chloroplast development and the effect of light suggests that photooxidative damage is responsible (van der Graaff et al., 2004). An essentially identical series of observations has been recorded during studies on both the *dov1* mutant (Kinsman and Pyke, 1998) and *30e5* (Brown, Fuglevand, Bilsland and Jenkins, unpublished work). Studies on the *cia1* mutant indicated that supplementing growth media with sucrose can increase leaf chlorosis - as can increasing the fluence rate of illumination. Although it seems likely now that alterations in a single gene have been studied from four independent perspectives, it is unclear how deficiencies in *de novo* purine biosynthesis lead to an enhancement in the susceptibility of chloroplasts to photooxidative damage.

6.7.4 An Increase in the Vulnerability of Chloroplasts to Photooxidative Damage May Account for a Series of Characteristics Common to Several Variegated / Chlorotic *Arabidopsis* Mutants, Including *30e5*

Initiation of photosynthesis in chloroplasts involves the capture of light energy by chlorophylls and the subsequent channelling of this energy, *via* the reaction centres of the photosystems, into the electron transport chain located in the thylakoid membrane. However, irradiation of the photosynthetic apparatus with an unmanageable quantity of light (which is not unusual in nature) results in leakage of electrons from excited chlorophylls and also from the electron transport system, and the production of reactive oxygen species (ROS). These ROS damage proteins, lipids, pigments, DNA and chloroplast components generally (Asada, 1996, Niyogi, 1999, Kimura et al., 2001). In extreme circumstances, such as plants deficient in compounds which counteract oxidative stress (eg. ascorbate or carotenoids), ROS can disrupt the ultrastructure of a chloroplast and destroy it (Yamamoto et al., 2000, Kimura et al., 2001).

The increase in chlorosis / pale-coloured leaf sector formation associated with malformed chloroplasts in mutants such as *30e5 / dov1*, *atd2 / cia1*, *cue1* and *im* (*immutans*) observed under high fluence rates suggests that photooxidative damage to chloroplasts is a common symptom in each of these plants (Wetzel et al., 1994, Streatfield et al., 1999, Rodermel,

2002). The genetic defects responsible for this phenotype in each case appear to be linked to chloroplast proteins. As described previously, the *ATD2 / CIA1* gene encodes ATasc2, *CUE1* encodes the phophoenolpyruvate / phosphate translocator of the chloroplast inner envelope (Streatfield et al., 1999) and IM is a plastid member of the alternative oxidase (AOX) class of inner mitochondrial membrane proteins (Wu et al., 1999). The similarity of IM to AOX suggests IM is a redox component of a phytoene desaturation pathway (Rodermel, 2002). As well as being critical for energy capture and functioning as storage organelles, plastids are involved in indispensable metabolic processes such as the biosynthesis of chlorophylls, carotenoids, purines, pyrimidines and fatty acids (Buchanan et al., 2000). Perhaps a deficiency in key metabolic processes within a plastid (eg. as found in the *atd2* mutant) is enough to disrupt its overall function. In the case of *immutans*, studies have highlighted a deficiency in phytoene desaturase (PDS) activity in the mutant. PDS, which can be inhibited by the herbicide norflurazon, is a key enzyme in the synthesis of carotenoids – and these protect the plant from chlorophyll mediated photooxidation (Oelmuller, 1989, Rodermel, 2002).

Damage to chloroplasts and the resulting reduction in photosynthetic capacity could account for the reductions in growth rate typically observed in variegated / chlorotic mutants. Moreover, accumulating data has suggested that signals sent to the nucleus by healthy plastids may be critical for proper leaf cell differentiation and for the expression of nuclear genes encoding components of the photosynthetic apparatus (Susek and Chory, 1992, Leon et al., 1998, Rodermel, 2002). A reduction in the expression of photosynthetic-related genes such as *CAB / Lhcb* is a common feature of many variegated / chlorotic mutants. Additionally, mutants such as *atd2*, *pac*, *cue1* and *im* show incomplete development of the palisade mesophyll cell layer (van der Graaff et al., 2004, Li et al., 1995, Streatfield et al., 1999, Rodermel, 2002). However, the fact that the *pale cress* mutant (*pac*) has normal *Lhcb* mRNA accumulation indicates that pathways transmitting a signal from the plastid to the nucleus which may be involved in regulating cellular development and photosynthetic gene

expression respectively, are separable (Reiter et al., 1994, Meurer et al., 1998, Rodermel, 2002).

The presence of the reticulate leaf phenotype, which has been observed in the *30e5 / dov1*, *atd2 / cia1* and *cue1* mutants, remains something of a mystery. Curiously, complementation of the reticulate leaf phenotype in *cue1* has been reported on addition of the aromatic amino acids (Streatfield et al., 1999).

6.7.5 It is Likely that the Extreme Sensitivity of the *30e5* Mutant to Supplementary UV-B Induced Growth Inhibition and Leaf Damage is a Result of the Increased Vulnerability of Chloroplasts to Photooxidative Damage

The common theme which unites the present work is an attempt to understand how *Arabidopsis* responds to UV-B radiation. Although not found to have an altered level of endogenous *CHS* gene expression, the *30e5* mutant showed arguably the greatest level of susceptibility to supplementary UV-B induced growth inhibition and leaf damage from amongst all the plants tested herein. However, *30e5* also showed an increase in the number of thymine dimers produced in response to UV-B, suggesting an increase in UV-B penetration into mutant plant tissue rather than simply an increase in oxidative stress (which might be expected to generate DNA breaks primarily - rather than thymine dimers). Nevertheless, UV-B can damage chlorophyll, carotenoids and the photosynthetic apparatus as well as generating ROS in plants, so perhaps the increased frailty of mutant plants could explain increased penetration of UV-B. Moreover, any damage to chloroplasts, which house enzymes of the shikimate pathway and provide chorismate for phenylpropanoid biosynthesis could result in a reduction in the quantities of UV-B protective sunscreen pigments (such as sinapate esters) being produced. It seems likely therefore, that the *30e5* lesion causes enhanced sensitivity to supplementary UV-B induced damage *via*, rather than independently of, its effect on chloroplasts. One method by which this hypothesis may be tested is to use the herbicide norflurazon (described above) to induce photooxidative stress in wild-type plants and

compare their susceptibility to injury induced by supplementary UV-B with that of untreated wild-type plants and the *30e5* mutant. Such a study is currently underway.

CHAPTER 7

FINAL DISCUSSION

7.1 Introduction

Responses of *Arabidopsis thaliana* plants to UV-B illumination were identified and investigated, genetic screens were developed and carried out and mutants were characterized. The aim of this chapter is to discuss and draw together the main conclusions arising from the work described overall and to summarise the prospects for future research.

7.2 Understanding Responses to UV-B

The title of this thesis is 'A Genetic Approach to Understanding the Responses of *Arabidopsis thaliana* to Ultraviolet-B Light.' The underlying objective was to investigate one or more of the several routes by which the UV-B signal is perceived and transduced to elicit responses in plants. As discussed in Chapter One, UV-B responses are manifold and complex and contrasting effects might even be observed, depending on factors such as UV-B fluence rate and plant developmental stage (Kim et al., 1998, Frohnmeyer and Staiger, 2003, Jenkins et al., 2001, Brosche and Strid, 2003). Thus 'positive' responses (such as photomorphogenesis), and 'negative' responses (such as macromolecular damage generated by UV-B induced reactive oxygen species) overlap and may be occurring simultaneously. The targets of this project were met by the development of screens for the isolation of UV-B photoregulatory mutants recorded in Chapter Three, the isolation and characterization of novel mutant alleles of the *UVR8* gene from a *CHS-Luc* reporter based screen described in Chapter Four, a screen for mutants deficient in UV-B induced phototropism detailed in Chapter Five and characterization of the *30e5* mutant which is presented in Chapter Six.

To summarise briefly then, the aim was to develop approaches for finding mutants by identifying clear responses to UV-B (Chapter Three) and to carry out screens (Chapter Four and Chapter Five) which would lead to mutant isolation (Chapter Four). Furthermore, mutants of *Arabidopsis* altered in their responses to UV-B were characterized and the corresponding genes identified (Chapter Four and Chapter Six). Ideally, this would result in the identification of one or more photoreception or signalling components involved specifically in UV-B responses (Chapter Four) and further insights into the regulation of the relevant signalling pathway(s) (Chapter Four).

7.3 A Genetic Approach to Investigating UV-B Responses in *Arabidopsis* has been Applied here with Differing Degrees of Success

Perhaps the biggest disappointment associated with the present work was the fact that no mutants lacking UV-B induced positive phototropic hypocotyl curvature were isolated from the screen described in Chapter Five. This screen proved to be more difficult technically than anticipated and it may be that plants are responding to UV-B over a very restricted range of fluence rates. For whatever reason, UV-B stimulated positive phototropic curvature was often not found initially in M_2 seedlings which were later identified as false positives (Sections 5.7 and 5.8). That is, the response was not completely uniform amongst non-mutant seedlings. It is also conceivable that mutants would have been isolated, were it not for a hidden redundancy in the roles adopted by the genetic components controlling UV-B induced phototropism. Perhaps there is more than one UV-B photoreceptor mediating phototropic hypocotyl bending.

By contrast, the *CHS-Luc* reporter gene was successfully used to isolate mutants of UV-B induced *CHS* gene expression (Chapter Four). It is worth noting, however, that the only underexpressing mutants found in the *CHS-Luc* screen correspond to four novel alleles of an existing mutant gene (*uvr8-1*) as illustrated in Sections 4.3.4 and 4.4.1 (Figures 4.11 and 4.13). The characterization studies presented in Chapter Four employ backcrossed *uvr8-1* and *uvr8-2* mutant lines. Clearly, a screen can only be successful in finding mutants if there are mutants to find. The repeated identification of the same mutant gene highlights the importance of UVR8 for UV-B induced *CHS* expression. Subsequent work has suggested a UV-B specific role for UVR8 (Figures 4.16, 4.17, 4.21 and 4.22), underlining both its significance and the likelihood that it functions in proximity to UV-B photoreception.

The *30e5* mutant was also isolated as one deficient in UV-B induced *CHS* gene expression *via* a reporter gene based screen (Fuglevand and Jenkins, unpublished work), although here *CHS-GUS* was used instead of *CHS-Luc*. However, whilst it has an increased susceptibility to supplementary UV-B induced growth inhibition and leaf damage (Figure 6.6), the *30e5*

mutant does not appear to be altered in endogenous levels of UV-B induced *CHS* transcript accumulation (Figure 6.5). The effect of the 30E5 gene product on UV-B responses is probably indirect. Recent work shows that the application of norflurazon (which inhibits the biosynthesis of carotenoids) to wild-type plants may mimic both the chlorotic phenotype exhibited by *30e5* and the increased susceptibility to damage generated by supplementary UV-B.

Significantly the *uvr8-1* mutant was initially isolated on the basis of its increased susceptibility to supplementary UV-B induced damage also (Kliebenstein et al., 2002). Thus screening for plants which are susceptible to damage caused by UV could result in the recovery of mutants which are linked either directly or indirectly to UV-B signal transduction. Consequently, mutant isolation may merely be an early step in identifying signalling components involved in a particular response. Extensive characterization of mutants is likely to be necessary before the significance of corresponding genes for a particular response can be appreciated. This truth is underlined by the fact that the more interesting mutant discovered here, apparently altered specifically in UV-B signal transduction (*uvr8*) is actually less susceptible to UV-B induced damage than is *30e5* – which is probably the consequence of a more general metabolic defect.

7.4 UVR8 Appears to Operate in a Signalling Pathway which is Specifically Concerned with Responses to UV-B

It has been observed that *uvr8-1* and *uvr8-2* mutant plants display a slight reduction in UV-A induced *CHS* gene expression compared with wild-type L. *er* controls. However this observation was almost overlooked, so great is the contrast between the level of *CHS* expression still produced in the mutants by UV-A and the total lack of a similar response under UV-B (Figure 4.16). Nevertheless, having found that *CHS* expression remains upregulated in response to cold treatment in *uvr8* mature leaf (also Figure 4.16) and far-red treatment in etiolated *uvr8* seedlings (Figure 4.17) and also that there is little difference between wild-type and mutant levels of *HY5* gene expression in response to treatments other than UV-B (Figures 4.21 and 4.22), we believe that UVR8 may operate in a signalling pathway which mediates responses specifically to UV-B.

Obviously, no matter how many non-UV-B responses are found to remain in the *uvr8* mutants, it is possible that other responses which have not yet been tested are significantly altered. A variety of ongoing work is geared to investigating the UV-B specificity of UVR8 regulated responses. The most recent experiment has provided evidence that the enhancement of *CHS* expression by addition of sucrose to growth media (Wade, 1999) is a response which is retained in the *uvr8-1* and *uvr8-2* mutant seedlings (data not shown). Therefore, the hypothesis that UVR8 is indeed concerned largely with UV-B responses continues to hold good.

7.5 The Roles of UVR8 and HY5 in *Arabidopsis*

One of the main themes of the work presented in Chapter Four is that two previously studied signalling components, each important in responses to light, have been found to be linked. Until now, very little information has been available on the UVR8 gene product and its mechanism of action still remains a mystery (Section 4.6.5). Nonetheless, we have shown that UVR8 appears to operate in a UV-B specific pathway and is necessary for UV-B induced expression of the *HY5* gene (Figure 4.22). Conversely, HY5 has been extensively studied and its function is more clearly understood (Section 4.6.4). HY5 is a pleiotropic transcription factor and its effects in *Arabidopsis* have considerable scope; it plays a role in inhibition of hypocotyl elongation (Ang and Deng, 1994), root development (Okada et al., 1998), gravitropism, cell elongation and proliferation, chloroplast development (Oyama et al., 1997) and auxin signalling (Cluis et al., 2004). Unlike UVR8, HY5 is not limited to UV-B responses and separate UV-A (Wade et al., 2001) and UV-B (Chapter Four) induced *CHS* expression pathways apparently converge on or before HY5. Most previous studies on the regulation of HY5 activity have focused on its proteasome-mediated degradation (Hardtke et al., 2000, Holm et al., 2001) but we have shown that control of *HY5* transcript accumulation is important for UV-B responses in mature *Arabidopsis* leaf tissue (Section 4.5). The *HY5* gene has previously been shown to be expressed in a phytochrome-dependent manner in etiolated seedlings after illumination (Quail, 2002a, Tepperman et al., 2001) but the UV-B induction of *HY5* gene expression in light grown seedlings does not depend on phyA or phyB (Ulm et al., 2004).

We have recently obtained results from a second microarray analysis of gene expression in mature *Arabidopsis* leaf tissue, this time examining the *hy5* mutant, which complements the study on *uvr8* presented in Chapter Four (Section 4.4.6). Both plants and data were treated in exactly the same way as previously and all gene lists were again cut at a false discovery rate of 5% to ensure that the results are comparable. Of 645 genes expressed in response to 4 hours of 3 $\mu Em^{-2}s^{-1}$ UV-B in mature *Arabidopsis* leaf tissue, 79 (including *HY5*) appear to

require UVR8 and 68 depend upon HY5. In total, 37 UV-B induced genes are normally expressed only in the presence of both UVR8 and HY5. It should be noted that the identification of a small number (approximately 5%) of these genes will be explicable as false positive results.

Amongst the thirty-seven UV-B induced genes which show deficiencies in expression level if either UVR8 or HY5 were removed, are a number which might play differing roles in protecting the plant from light induced damage (see Section 4.6.3). These include flavonoid biosynthesis genes (such as chalcone synthase, flavonol synthase, chalcone isomerase, flavanone 3-hydroxylase and dihydroflavonol 4-reductase), ELIPs and the CPD photolyase gene *PHR1*. Intriguingly, a gene encoding the putative photoreceptor *CRYD* (Kleine et al., 2003) also appears to be expressed under UV-B *via* a signalling pathway which involves both UVR8 and HY5. Failure to induce these UVR8 / HY5 regulated genes in response to UV-B results in inhibition of growth and damage to plants treated with supplementary UV-B (Figure 4.23). It is also clear that UVR8 regulates the UV-B induced expression of another bZIP transcription factor gene, *HYH* (Table 4.19), the role of which may overlap with that of *HY5* in UV-B responses (Ulm et al., 2004).

It is noteworthy that a number of the genes (eg. *CRYD*, *HY5*, *HYH* and the *ELIP*s), which were found to be upregulated by UV-B *via* UVR8 in mature leaf (Table 4.19) were also identified as being induced by long wavelength UV-B (>305 nm) treatment of seven day old seedlings in a study conducted by Ulm *et al.* (Ulm et al., 2004). Here, an extensive examination of the gene expression changes produced by UV-B of greater than 305 nm, 295 nm or approximately 285 nm in wavelength respectively, was carried out using white light grown *Arabidopsis* seedlings. A short, 15 minute UV-B treatment was given in each case before the seedlings were returned to white light and harvested either one hour (to examine short term changes) or six hours after the start of the UV-B treatment for microarray analysis (Ulm et al., 2004). It was found that the long wavelength (>305 nm) UV-B-induced changes in seedling gene expression were generally short lived (present at one but not six hours post-irradiation) compared to transcript level changes induced by UV-B which included the shorter

wavelengths as well (eg. >285 nm approximately). Therefore, it was suggested that long wavelength (>305 nm) UV-B might be detected by a specific UV-B perception and signalling mechanism. Furthermore, Ulm and co-workers found that some of the genes induced by >305 nm UV-B at one hour post-irradiation are actually repressed when the wavelength range is simply extended by using a >285 nm UV-B treatment instead. One possible explanation is that the antagonistic downregulation of gene expression might represent the activity of a second UV-B signalling pathway – perhaps resulting from the effects of UV-B induced cellular stress. However, parallel studies using the CPD photolyase deficient *uvr2* mutant provided no evidence that either pathway was mediated *via* damage to DNA (Ulm et al., 2004).

The results presented in this thesis indicate that UVR8 is a key component of a UV-B specific signalling pathway which stimulates the expression of more than seventy genes. The increased sensitivity of the *uvr8* mutant to supplementary UV-B induced growth inhibition and leaf damage shows that the gene product is likely to be essential for survival in sunlight, whereas no deleterious effects are apparent in *uvr8* mutant plants grown in the absence of UV-B (see Figure 4.10). It has further been shown that HY5 plays crucial part in implementing responses to the UV-B signal transduced by UVR8, since approximately half of the genes upregulated *via* UVR8 are also dependent upon HY5. These results highlight a novel role for HY5: protecting established plants from ultraviolet light. Not only is HY5 required for the normal development of the photosynthetic machinery, but it also helps to maintain photosynthetic function by effecting UV-B protection.

7.6 Conclusions

From the work presented in this study which takes a genetic approach to investigating the responses of *Arabidopsis thaliana* to UV-B a number of conclusions can be drawn: (i) Great care must be taken to design practical screens for mutants of interest. (ii) The UVR8 gene product is required for UV-B induced upregulation of a series of genes including many of those involved in flavonoid biosynthesis, such as *CHS*. (iii) UVR8 appears to function specifically in a UV-B regulated photoreception / signalling pathway and is required for UV-B induced transcript accumulation of the *HY5* gene. (iv) The expression of a subset of genes important in UV-B photoprotection (eg. *ELIP*s, *PHR1* and genes of the flavonoid biosynthetic pathway) is stimulated by a signalling pathway which requires both UVR8 and HY5. (v) Nevertheless, a great many UV-B induced genes are expressed even in the absence of UVR8 and HY5. (vi) Positive phototropic hypocotyl curvature appears to be stimulated or enhanced by UV-B even when the phototropins are removed. (vii) The *30e5* mutant is more susceptible to supplementary UV-B induced growth inhibition and leaf damage than are *uvr8*, *fah1*, *tt4* or *tt5* mutants. (viii) The *30e5* mutant gene is an allele of *DOV1 / F196* and probably an allele of the *ATD2 / CIA1* gene. Note that *ATD2 / CIA1* encodes ATase2, an enzyme catalyzing the first committed step of *de novo* purine biosynthesis in *Arabidopsis*.

7.7 Future Work

7.7.1 Prospects for the Genetic Approach in Understanding UV-B Responses of *Arabidopsis*

The *Arabidopsis* UV-B response which provided the basis for the successful screen described in Chapter Four was *CHS* gene expression. One of the reasons this particular response was selected for study is that it appears to be mediated independently of UV-B signalling pathways (eg. those involving jasmonate and reactive oxygen species) which overlap with wound-response and pathogen-defence (Jenkins et al., 2001). Despite our confidence that a number of UV-B signalling components might be identified as a result of this project, four new alleles of a previously identified gene (*UVR8*) emerged as the sole products after a screen for underexpressing mutants of UV-B induced *CHS* gene expression. Whilst underlining the significance of the UVR8 gene product for UV-B photoreception / signal transduction in plants, this finding suggests that further pursuit of mutants deficient in UV-B induced *CHS* gene expression is unlikely to be fruitful. It is however, possible that the overexpressing mutants isolated during the screen (*chum2* and *chum35*) may signify important negative regulators of the UV-B response and these mutants might be worthy of further characterization.

It is possible too, that UV-B specific components of *CHS* expression remain undiscovered because other gene products have an overlapping role. Such redundancy exposes one of the limitations of the genetic approach (discussed in Section 1.6.5). A potential answer to the problem of redundancy in genetic screens is to try to activate rather than deactivate genes of interest; this can be done by introducing T-DNA vectors containing transcriptional enhancers – such as that from the cauliflower mosaic virus 35S gene (Weigel et al., 2000). Activation tagging has already proved to be a valuable 'mutant-mining tool' in *Arabidopsis* (Nakazawa et al., 2003, Borevitz et al., 2000) and could provide further insights into the regulation of UV-B induced responses. Activation tagging has the advantage of making gene identification easier than does EMS mutagenesis. As noted previously (see Section 1.6.4), map-based

cloning remains a labour-intensive and demanding procedure despite recent advances in plant molecular genetics.

It is also of significance that the majority of UV-B induced genes highlighted by the microarray analyses are apparently regulated neither by UVR8 (see Table 4.20) nor by HY5. Note that because the microarray analyses were not extended to include *hy5* mutant control plants grown continuously under non-inducing light conditions, we cannot apply the same level of stringency in identifying genes which are UV-B induced independently of HY5 as we can for those which do not require UVR8 (Table 4.20). It is worth noting that non-UVR8 regulated UV-B induced genes could be used to perform further screens for mutants altered in UV-B responses in the same way as the *CHS* gene has successfully been used here. If upstream signalling components of other UV-B induced pathways can be identified then microarray analyses with the corresponding mutants might identify subsets of genes regulated in the different pathways.

7.7.2 Investigating How UVR8 Controls UV-B Responses

As described in Section 4.6.5, the mechanism by which UVR8 mediates responses to UV-B in *Arabidopsis* is presently unclear. One of the key questions which this work has not answered is: does a typical photoreceptor (eg. a flavoprotein) mediate UV-B induced *CHS* gene expression? Despite this shortcoming, it is extremely curious that just such a putative photoreceptor, cryD (Kleine et al., 2003), occurs prominently amongst our microarray data which highlights UVR8 and HY5 regulated UV-B induced genes. It is just possible that cryD is the UV-B photoreceptor which mediates *CHS* gene expression *via* UVR8 and HY5 even though it is itself upregulated by these signalling components. Perhaps cryD normally occurs at very low levels in the plant cell but in response to UV-B it upregulates its own expression, together with that of other UV-B induced genes, to increase flux through the pathway as a whole. This hypothesis would certainly explain the otherwise enigmatic presence of a putative photoreceptor near the top of our lists of UV-B induced genes. cryD (At5g24850), like the cryptochrome and phototropin photoreceptors, both binds flavin adenine dinucleotide (FAD)

and lacks photolyase activity. Furthermore, cryD has been shown to be capable of associating with DNA and to possess an N-terminal sequence which mediates import into chloroplasts and mitochondria (Kleine et al., 2003). One way to examine the possibility that cryD is our elusive UV-B photoreceptor would be to investigate whether UV-B induced *CHS* gene expression is lost in a *cryD* mutant, although the fact that a *cryD* mutant was not found in the *CHS-Luc* screen suggests that expression is unlikely to be lost – unless mutations in the *CRYD* gene are lethal. The possibility that mutations in UV-B signalling components may jeopardize the very survival of seedlings acts as a reminder that it is conceivable that UV-B induced *CHS* expression requires several other proteinaceous factors and that the pathway is neither short nor encumbered with redundant components.

As already described (Section 4.6.5) a variety of studies are underway which aim to elucidate the mechanism by which UVR8 operates, although it appears to share only one significant functional feature with RCC1 despite extensive sequence similarity. The feature in question is that both RCC1 and UVR8 bind histones (Cloix and Jenkins, unpublished work). Perhaps yeast-two-hybrid or analogous studies might identify signalling partners; alternatively, a screen for mutants which suppress the *uvr8* lesion might constitute a fruitful approach to identifying UVR8 interacting components.

In conclusion, the present work has highlighted the significance of UVR8 for UV protection and its involvement in the regulation of a number of genes with a variety of photoprotective roles. The specific association of the UVR8 gene product with UV-B responses is demonstrated and it is shown that UVR8 appears to operate separately from pathways controlling the same responses (eg. *CHS* expression) but stimulated by other light qualities (eg. UV-A and far red) in *Arabidopsis thaliana*. The UV-B induced upregulation of the key transcription factors HY5 and HYH has also been shown to occur *via* UVR8 at the transcript level and a second microarray involving the *hy5* mutant has identified a subset of UV-B induced genes which depend upon both UVR8 and HY5. Further work will be necessary to realize the full extent of the significance of UVR8 for UV-B responses in plants.

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