UV/BLUE LIGHT SIGNAL TRANSDUCTION REGULATING GENE EXPRESSION IN *ARABIDOPSIS*

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John M. Christie

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Department of Biochemistry and Molecular Biology,

University of Glasgow

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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PREFACE

Aspects of the work presented in this thesis have recently been, or are due to be, published. The majority of data presented in chapter 4 is published in Christie and Jenkins (1996). Distinct UV-B and UV-A/blue light signal transduction pathways induce chalcone synthase gene expression in *Arabidopsis* cells. *Plant Cell* **8**, 1555-1567. J.M. Christie is also an author of the following papers: Jenkins *et al.* (1995). Plant responses to UV and blue light: biochemical and genetic approaches. *Plant Sci.* **112**, 117-138; Jenkins *et al.* (1996). UV-B perception and signal transduction. In *Plants and UV-B*; ed. P.J. Lumsden. Cambridge University Press, in press; Jenkins *et al.* (1996). Biochemical and genetic approaches to UV/blue light signal transduction. In *Proceedings of the 12th International Congress on Photobiology*. OEMF SpA, Milan, in press.

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ABBREVIATIONS

Abbreviations not described in the 'Instructions to Authors', Biochemical Journal (1992) **281**, pp. 1-19, are listed below along with their definitions.

blu	blue light uninhibited
CAB	LHCB1 chlorophyll-binding protein
CAT	chloramphenicol acetyltransferase
CCCP	carbonyl cyanide-*m*-chlorophenylhydrazone
C4H	cinnamate 4-hydroxylase
CHI	chalcone isomerase
4CL	4-coumarate:CoA ligase
CHS	chalcone synthase
cop	constitutive photomorphogenic
cpm	counts per minute
CPRF	common plant regulatory factor
CRY	cryptochrome
CsCl	caesium chloride
DAG	diacylglycerol
DCCD	N,N'-dicyclohexylcarbodi-imide
DEPC	diethyl pyrocarbonate
det	de-etiolated
DFR	dihydroflavonol reductase
DMSO	dimethyl sulphoxide
ds	double stranded
EGTA	ethyleneglycol-bis-(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
FDA	fluorescein diacetate
fhy	far-red elongated hypocotyl
fnr	ferridoxin $NADP^+$ oxidoreductase
fus	*fusca*
G_i	inhibitory G protein
GUS	β-glucuronidase
HFR	high fluence response
HRP	horseradish peroxidase

hp	high pigment
hy	long hypocotyl
icx	increased chalcone synthase expression
IP_3	inositol 1,4,5-trisphosphate
LFR	low fluence response
lh	long hypocotyl
lip	light-independent photomorphogenesis
LRU	light regulatory unit
MBP	maltose-binding protein
ML-7	1-(5-iodonapthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine
ML-9	1-(5-chloro-napthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine
MLCK	myosin light chain kinase
nph	non-phototropic hypocotyl
NPPB	5-nitro-2,3-phenylpropylaminobenzoic acid
OAc	acetate
OD	optical density
PAA	phenylacetic acid
PAL	phenylalanine ammonia-lyase
PEG	polyethylene glycol
Pfr	form of phytochrome which absorbs maximally far-red light
pH	hydrogen ion concentration ($-\log_{10}$)
PHY	phytochrome
PIP_2	phosphatidylinositol 4,5-bisphosphate
PMA	phenylmercuric acetate
PP	protein phosphatase
Pr	form of phytochrome which absorbs maximally red light
PR	pathogenesis-related
psi	pounds per square inch
PVP	polyvinyl pyrolidone
rbcS	ribulose-1,5-bisphosphate carboxylase small subunit
ROS	reactive oxygen species
35S	cauliflower mosaic virus 35S promoter
SD	standard deviation of the mean
TCH	touch
TEA	tetraethylammonium
UV-A	320-390 nm
UV-B	280-320 nm
UV-C	wavelengths below 280 nm
VLFR	very low fluence response

v/v	volume/volume
W-7	*N*-(6-aminohexyl)5-chloro-1-naphthalenesulphonamide
W-5	*N*-(6-aminohexyl)-1-naphthalenesulphonamide
w/v	weight/volume

SUMMARY

Plants have evolved a number of mechanisms to protect themselves against environmental stresses. For example, in response to potentially harmful levels of UV light plants can accumulate flavonoids which, among other functions, act as UV-protective pigments. Chalcone synthase (CHS) is the key enzyme which commits the phenylpropanoid pathway to flavonoid biosynthesis. As the expression of *CHS* and other flavonoid biosynthesis genes is controlled by UV/blue light in higher plants, these genes provide ideal subjects for investigation of the cellular and molecular mechanisms involved in coupling UV/blue photoreception to transcription.

To investigate the signal transduction processes concerned with the induction of *CHS* expression by UV/blue light, the effects of specific agonists and inhibitors were examined in a photomixotrophic *Arabidopsis* cell suspension culture. This cell culture behaves similarly to mature *Arabidopsis* leaves in the light regulation of *CHS*. That is *CHS* transcript levels are induced by UV-B and UV-A/blue light but not phytochrome. Moreover, experiments with a *hy4* mutant cell suspension culture demonstrate that the effects of UV-B and UV-A/blue light on *CHS* expression are mediated by separate detection systems, the latter involving the CRY1 photoreceptor.

Pharmacological studies with the *Arabidopsis* cell culture indicate that the UV-B and UV-A/blue phototransduction processes require cellular calcium. The inhibitors used suggest that an intracellular pool of calcium may be involved. However, the artificial elevation of cytosolic calcium using an ionophore is insufficient on its own to stimulate *CHS* expression. Similarly, in preliminary experiments with transgenic *Arabidopsis* containing cytosolic aequorin, no significant rapid increase in calcium is observed in response to either UV-B or UV-A/blue light. Possible reasons for this discrepancy are discussed.

The UV-B induction of *CHS* expression appears to involve calmodulin because it is strongly inhibited by the antagonist W-7. In contrast, W-7 has little, if any, effect on

the UV-A/blue response, indicating that the UV-B and UV-A/blue light signal transduction pathways regulating *CHS* are, at least in part, distinct. Further evidence shows that both pathways involve protein phosphorylation and require cytoplasmic protein synthesis. The involvement of chromophore excitation, ion fluxes and heterotrimeric G proteins in UV/blue light signal transduction have also been investigated.

From the above pharmacological experiments, it is evident that the UV-B and UV-A/blue phototransduction pathways are distinct from the phytochrome signal transduction pathway regulating *CHS* expression in other species. Support for this conclusion comes from the observation that activators/inhibitors of phytochrome signalling have no effect on *CHS* expression in the *Arabidopsis* cell culture. Moreover, photoreception by phytochrome results in a transient increase in *CHS* transcripts, whereas UV/blue photoreception effects a sustained increase.

A transient gene expression system in protoplasts derived from the *Arabidopsis* cell culture was developed to identify the *cis*-regulatory elements required for the UV-B and UV-A/blue light induction of *CHS*. Light-regulated transient expression of β-glucuronidase (GUS) driven by the full-length *Arabidopsis CHS* promoter has been optimised using a modified polyethylene glycol transfection protocol. Similar to the expression pattern found in *Arabidopsis* cells, *CHS* transcript accumulation and *CHS* promoter activity are induced specifically by UV-B and UV-A/blue light in cell culture protoplasts. Transient expression of various *CHS* promoter-GUS fusions shows that a light regulatory unit (LRU), equivalent to the parsley LRU1, is neccessary and sufficient to mediate both UV-B and UV-A/blue light-induced GUS activity. Thus the UV-B and UV-A/blue phototransduction pathways terminate with an effect on the biogenesis and/or activity of transcription factors that bind to the LRU region of the *Arabidopsis CHS* promoter. The effects of specific inhibitors on the UV-B and UV-A/blue light induction of *CHS* promoter activity in *Arabidopsis* protoplasts have also been examined. The results are consistent with the effects observed at the transcript level in the *Arabidopsis* cell culture. However, in contrast to their effects in

Arabidopsis cells, several inhibitors appear to cause a general inhibition of transcription and/or translation activities in cell culture protoplasts. Possible reasons for these observations are discussed.

Phenylalanine ammonia-lyase (PAL) is the first step in the general phenylpropanoid pathway which subsequently branches to the synthesis of flavonoids. Like *CHS*, *PAL* gene expression is induced by UV-B and UV-A/blue light in the *Arabidopsis* cell culture. Jasmonic acid, a mediator of plant defense against pathogen attack, also stimulates *PAL* expression but not *CHS*. This is consistent with the lack of *CHS* induction by fungal elicitors in *Arabidopsis* and parsley cells. While protein phosphatase inhibitors strongly attenuate the UV/blue light stimulation of *CHS* in the *Arabidopsis* cell culture, *PAL* gene expression is induced by phosphatase inhibitors, UV/blue light or both. This mode of regulation is very similar to the effects of fungal elicitor on the light regulation of *PAL* and *CHS* expression in parsley cells. Moreover, pharmacological studies show that the phosphatase inhibitor, cantharidin, activates an early event in a signalling pathway coupled to *PAL* gene expression which involves an influx of calcium, cytoplasmic protein synthesis and protein phosphorylation. It is therefore proposed that protein phosphatase inhibitors mediate their effects on *PAL* and *CHS* expression in the *Arabidopsis* cell culture through a pathway that also mediates the elicitor response. Evidence for this hypothesis is discussed.

CHAPTER 1: PHOTOSENSORY PERCEPTION AND SIGNAL TRANSDUCTION IN PLANTS

1.1 Introduction

Environmental factors have an extensive regulatory influence on plant growth and development. Perhaps the most important environmental factor is light. Light is not only a substrate for photosynthesis but a stimulus that regulates numerous developmental and metabolic processes, including seed germination, leaf development, chloroplast biogenesis, the inhibition of hypocotyl elongation, flowering and anthocyanin formation (Jenkins, 1991). Collectively, these light-dependent processes are known as photomorphogenesis.

In higher plants, photomorphogenesis is controlled by several classes of photoreceptors. These are the phytochromes, which monitor the red and far-red regions of the spectrum, the UV-A/blue light receptors and the putative UV-B receptor(s) (Quail, 1994). Light signals perceived by these photoreceptors are transduced via signalling components to bring about the diverse downstream physiological responses associated with photomorphogenic development. In many cases, reponses have been shown to involve the transcriptional regulation of specific genes (Terzaghi and Cashmore, 1995a). Some genes, such as many involved in photosynthesis, are induced by light (Gilmartin *et al.*, 1990). Other genes, such as those encoding phytochrome A, become repressed upon exposure to light (Thompson and White, 1991).

In recent years considerable progress has been made in defining the photoreceptors that mediate the effects of light and the *cis*-elements and transcription factors that are concerned with the photoregulation of gene expression. However, our understanding of the signal transduction processes that couple photoreception to transcription remains fragmentary. As reviewed in this chapter, recent advances made through the application of biochemical and molecular genetic approaches are beginning

to provide important insights into the complex mechanisms associated with photosensory perception and signal transduction in plants.

1.2 The Phytochromes

1.2.1 The phytochrome photoreceptor

It is well established that responses to red and far-red light are mediated by the photoreceptor phytochrome (Furuya, 1993; Quail *et al.*, 1995). Phytochrome is a cytosolically localised protein dimer consisting of two approximately 120 kDa polypeptides, each carrying a covalently bound linear tetrapyrrole chromophore. The photosensory function of this molecule depends on its ability to exist in two photo-interconvertible forms: one (Pr) which absorbs maximally red light and one (Pfr) which absorbs maximally far-red light. Absorption of red light converts Pr to Pfr, which is generally considered to be the active form of phytochrome. Thus the formation of Pfr correlates with the induction of many physiological and gene expression responses. Pfr can be converted back to Pr by an immediate illumination with far-red light. Consequently, red light-induced responses mediated by phytochrome are typically reversible by far-red light. This red/far-red photoreversibility is a classic test for the involvement of phytochrome in a particular response.

Contrary to earlier assumptions that phytochrome is a single molecular species, it is now apparent that, in most plant species, phytochrome is encoded by a small multigene family. For example, five phytochrome genes have been identified in *Arabidopsis*, designated *PHYA*, *B*, *C*, *D*, and *E* (Sharrock and Quail, 1989). These different phytochrome species can be distinguished by their relative abundance and stability as Pfr (Quail, 1994). PHYA is the most abundant phytochrome in etiolated (dark-grown) seedlings, but is unstable as Pfr. On the other hand, PHYB and PHYC are more stable than PHYA in the Pfr form and are present at similar levels in light and dark-grown plants. Since PHYA protein levels decrease in the light, PHYB and PHYC

(and possibly PHYD and PHYE) are the predominant phytochrome species in light-grown plants.

1.2.2 Phytochrome-mediated responses

Although the range of responses regulated by phytochrome is diverse, they can be separated into three different classes based on their fluence requirements (Mancinelli, 1994): low fluence responses (LFRs), very low fluence responses (VLFRs) and high irradiance responses (HIRs). LFRs represent classical phytochrome-mediated responses: these are activated by red light and exhibit red/far-red photoreversibilty. For a LFR to occur, a high amount of Pfr is required but only for a relatively short period of time. Red light (1-1000 μmol m^{-2}) is estimated to convert 80% of Pr to Pfr whereas subsequent irradiation with far-red light results in a phytochrome equilibrium of about 97% Pr and 3% Pfr. In contrast, VLFRs require as little as 0.1 nmol m^{-2} red light for induction (estimated to convert 0.01% of Pr to Pfr). Furthermore, VLFRs can be induced by far-red treatments alone and therefore do not exhibit red/far-red photoreversibility. The explanation for this is that Pr can also weakly absorb far-red light, so that a small percentage is converted to Pfr (2-3%). HIRs typically require a continuous illumination of relatively high fluence (>10 μmol m^{-2}) and tend to be more efficiently induced by far-red light. As described below, recent transgenic and genetic approaches demonstrate that individual members of the phytochrome family have specialised regulatory roles in controlling various types of responses under different light qualities.

1.2.3 Photoregulatory mutants

Photoregulatory mutants have provided important insights into the mechanisms involved in plant photoreception and signal transduction (Chory, 1993; Jenkins *et al.*, 1995). Some of the first photoregulatory mutants were isolated by screening for an

elongated hypocotyl in white light. These mutants include the *lh* (*l*ong *h*ypocotyl) mutant in cucumber (Adamse *et al.*, 1987), *aurea* mutants of tomato (Adamse *et al.*, 1988) and the *hy* (long *hy*pocotyl) mutants of *Arabidopsis* (Koornneef *et al.*, 1980; Chory *et al.*, 1989a; Parks and Quail, 1993). In *Arabidopsis*, there are at least seven mutant loci that lead to the loss of photoinhibition of hypocotyl elongation. Three of these mutants (*hy1*, *hy2* and *hy6*) are believed to correspond to genes required for phytochrome chromophore biosynthesis or attachment and are probably largely deficient in all phytochromes (Chory *et al.*, 1989a). The *hy4* mutant is specifically altered in the blue light-dependent inhibition of hypocotyl growth (Koornneef *et al.*, 1980) and has recently been shown to encode a UV-A/blue photoreceptor (Ahmad and Cashmore, 1996). On the other hand, *hy5* is primarily altered in red and far-red light-induced hypocotyl suppression (Koornneef *et al.*, 1980). To a lesser degree, *hy5* is also insensitive to blue light. Since *hy5* seedlings have normal levels of spectrophotochemically active phytochrome, they are probably not photoreceptor mutants. It is therefore thought that *HY5* encodes a common signal transduction component that effects both red/far-red light and blue light-mediated hypocotyl responses.

1.2.4 Functions of different phytochromes

Phytochrome-deficient mutants and phytochrome overexpressors are revealing specialised roles for individual phytochromes in photomorphogenesis (Quail *et al.*, 1995). Recent verification that the *HY3* locus encodes the PHYB apoprotein has uncovered numerous roles for PHYB (Reed *et al.*, 1993). Among these, PHYB is the primary photoreceptor controlling responses to continuous red light (LFRs). The *hy3* mutant exhibits extended hypocotyls in continuous white or red light (Reed *et al.*, 1993) but is unaltered in this response to continuous far-red light (McCormac *et al.*, 1993). Moreover, transgenic *Arabidopsis* seedlings overexpressing the PHYB coding sequence, using the cauliflower mosaic virus 35S promoter, show an enhanced

sensitivity to red light but exhibit wild-type responsiveness to continuous far-red light (McCormac *et al.*, 1993).

Further genetic evidence suggests that PHYA has a distinct functional role in relation to PHYB. Parks and Quail (1993) recently identified a new class of *Arabidopsis* long hypocotyl mutants. These mutants were isolated by screening for an elongated hypocotyl in continuous far-red light. Mutants with an altered ability to respond to red light were discarded because they probably suffered from a general defect in phytochrome signalling, such as chromophore biosynthesis. The remaining mutants, designated *hy8*, were subsequently shown to be lesions in the *PHYA* gene (Dehesh *et al.*, 1993). The *hy8* phenotype indicates that PHYA is necessary for continuous far-red light perception (the so-called far-red HIR). While *hy8* lacks the ability to respond to continuous far-red light, it exhibits wild-type responsiveness to continuous red or white light (Parks and Quail, 1993). In addition, transgenic plants overexpressing PHYA show an enhanced sensitivity to continuous far-red light (McCormac *et al.*, 1993).

Whitelam *et al.* (1993) reported the identification of additional *Arabidopsis* mutants that display an elongated hypocotyl in far-red but not white light. These *fhy* (*f*ar-red elongated *hy*pocotyl) mutants represent three mutant loci. Similar to the *hy8* mutants identified by Parks and Quail (1993), *fhy2* mutants were shown to contain structural alterations within the *PHYA* gene. In contrast, *fhy1* and *fhy3* mutants have normal levels of functional PHYA. Thus, *FHY1* and *FHY3* gene products may be responsible for the transduction of the far-red light signal from PHYA to downstream processes involved in the regulation of hypocotyl growth. Whether phytochromes C, D, and E have distinct physiological functions is yet to be determined.

1.3 The UV-A/blue Photoreceptors

1.3.1 UV-A/blue light responses

Although many aspects of photomorphogenesis are controlled by phytochrome, numerous responses are regulated by the near UV (UV-A; 320-390 nm) and blue (390-500 nm) regions of the spectrum. Examples include the inhibition of hypocotyl elongation, stomatal opening, phototropism and the transcription of various genes (Kaufman, 1993; Short and Briggs, 1994; Jenkins *et al.*, 1995). UV-A/blue light-mediated responses are not only present in higher plants but are commonly found in lower organisms. For instance, blue light controls phototropism in *Phycomyces* sporangiophores (Galland and Lipson, 1987), morphogeneic events in algae (Senger and Schmidt, 1994) and gene expression in the photosynthetic bacterium *Rhodobacter sphaeroides* (Shimada *et al.*, 1992).

The action spectra for many UV-A/blue light-initiated processes have been obtained (Briggs and Iino, 1983). Comparison of these action spectra reveal considerable variation, implying that several different UV-A/blue photoreceptors exist in plants. Despite this, similar action spectra can be recognised for certain responses, suggesting some similarity between the photoreceptors involved. A number of blue light responses have action spectra resembling the absorption spectra of flavoproteins. Therefore, it is generally accepted that blue light receptors contain a flavin chromophore (Briggs and Iino, 1983; Galland and Senger, 1988a; Short and Briggs, 1994). Support for this hypothesis has come from biochemical experiments using compounds that interact with flavins (Briggs and Iino, 1983; Galland and Senger, 1988a; Warpeha *et al.*, 1992). However, several action spectra cannot be explained easily by the flavin hypothesis. Further evidence indicates that pterins can function as either the sole chromophore pigment or an accompanying chromophore pigment for UV-A/blue photoreception (Galland and Senger, 1988b; Schmidt *et al.*, 1990). More recently, Quiñones and Zeiger (1994) have reported that the carotenoid, zeaxanthin, may be a choromophore mediating blue light-induced phototropism in maize coleoptiles. Hence

the presence of several chromophore types and their possible combinations may account for the variabilty in action spectra observed for UV-A/blue light-mediated responses in plants. Recent identification and characterisation of the first UV-A/blue light receptor from any organism has confirmed that flavins play an important role in UV-A/blue photoreception (Ahmad and Cashmore, 1996).

1.3.2 The CRY1 photoreceptor

1.3.2.1 *Arabidopsis* blue light mutants

As mentioned previously, the *hy* mutants of *Arabidopsis* were isolated by screening for a long hypocotyl in white light (Koornneef *et al.*, 1980). While a number of these mutants were found to be deficient in particular phytochrome molecules (Chory, 1992; Chory, 1993), hypocotyl extension of the *hy4* mutant was inhibited by red and far-red light, indicating a normal response to phytochrome. On the other hand, *hy4* showed a reduced level of inhibition by blue light (Ahmad and Cashmore, 1993), indicating that it was specifically altered in blue light perception. Further characterisation revealed that the *hy4* mutant has a longer hypocotyl phenotype in UV-A and green light (Jenkins *et al.*, 1995; Lin *et al.*, 1995a).

Liscum and Hangarter (1991) isolated three additional loci by screening for *Arabidopsis* mutants with a long hypocotyl in blue light. Three of these mutants lack blue light-mediated hypocotyl repression and were designated *blu1*, *blu2* and *blu3* (*b*lue *l*ight *u*ninhibited). However, further genetic analysis revealed that the *blu* mutants are in fact alleles of *hy4* (Jenkins *et al.*, 1995; Ahmad and Cashmore, 1996).

The double mutant phenotypes between blue light-deficient and red light-deficient mutants, support the notion that blue light and phytochrome detection systems are distinct. Double mutants made between *hy4* and the phytochrome-deficient mutants, *hy1*, *hy2* and *hy6*, exhibit a hypocotyl length that is the sum of the corresponding single mutant phenotypes (Koornneef *et al.*, 1980). Furthermore, plants homozygous

for *blu1* and *hy6* have very long hypocotyls and closed cotyledons characteristic of etiolated (dark-grown) seedlings, suggesting that most, if not all, de-etiolation responses are mediated by either phytochrome or blue light receptors (Liscum and Hangarter, 1991).

1.3.2.2 Cloning of the *HY4* locus

The *HY4* gene was isolated through the availability of a *hy4* mutant allele tagged with a T-DNA insertion (Ahmad and Cashmore, 1993). Examination of the derived amino acid sequence revealed that the *HY4* gene encodes a 75 kDa protein with significant homology to microbial DNA photolyases, which catalyse the light-dependent repair of DNA. Interestingly, homology was highest in regions known to be involved in photolyase chromophore binding. The C-terminal domain binds a flavin adenine nucleotide (FAD) molecule whereas the N-terminal domain, depending on the photolyase, binds either a pterin (short wavelength absorbing) or a deazaflavin derivative (long wavelength absorbing) (Sancar, 1994). The HY4 protein was found to mostly resemble the latter, longer wavelength absorbing type of photolyase. However, it was suggested that the HY4 protein itself may not function as a photolyase since it lacks a conserved tryptophan residue thought to be required for the recognition of pyrimidine-dimers. Interestingly, the C-terminal third portion of the HY4 sequence shows considerable homology to rat smooth-muscle tropomyosin A. This region, not found in photolyases, is believed to be functionally important and may be involved in initiating signal transduction rather than photoreception (Ahmad *et al.*, 1995). The hydrophobicity plot of HY4 reveals no obvious membrane-spanning domain suggesting that the protein is soluble. Northern analysis demonstrates that *HY4* gene expression is not light-regulated and transcripts are present in stems, leaves, flowers, siliques and to a lesser extent in roots. Cashmore and co-workers subsequently re-named the HY4 protein as CRY1, after cryptochrome, the term previously used to describe plant UV-A/blue photoreceptors (Ahmad and Cashmore, 1996).

1.3.2.3 DNA photolyases

The characterisation of CRY1 has been greatly facilitated by the extensive work done on microbial DNA photolyases (Sancar, 1994). These enzymes catalyse the UV-A/blue light-dependent repair of pyrimidine dimers in DNA damaged by UV light in a process known as photoreactivation. Photolyases absorb light energy by the action of a primary light-harvesting chromophore bound non-covalently in the N-terminal region of the protein. Short wavelength type photolyases (absorbing maximally at 350-370 nm) bind a pterin as the primary light-harvesting chromophore whereas the long wavelength type (absorbing maximally at 450 nm) bind a deazaflavin. In each case, light energy is transferred to a second chromophore, flavin adenine nucleotide (FAD), bound non-covalently in the C-terminal region of the enzyme. The photoreduced FAD molecule then provides the energy required for the cleavage of the pyrimidine dimer. It is generally considered that both chromophores are required for DNA repair although light absorption by either the deazaflavin/pterin or FAD molecule can result in the excision of pyrimidine dimers (Jorns *et al.*, 1990). These enzymes therefore provide interesting comparative models for the study of plant UV/blue photoreceptors.

1.3.2.4 Characterisation of CRY1

In order to characterise CRY1 in more detail, Lin *et al.* (1995b) expressed and purified the 75 kDa protein from insect cells transfected with recombinant baculovirus containing the *HY4* coding sequence. Purified CRY1 is a yellow protein with an absorption spectrum resembling that of a flavoprotein. As predicted from the primary amino acid sequence (Ahmad and Cashmore, 1993), the protein had no apparent photolyase activity *in vitro*. In addition, the expression of CRY1 could not rescue a photolyase deficient mutant of *E. coli*. These observations are consistent with the earlier suggestion that CRY1 is a non-photolyase, flavin-type photoreceptor. Indeed, CRY1 noncovalently binds stoichiometric amounts of oxidised FAD. Under anaerobic conditions, the flavin chromophore bound to CRY1 could be photoreduced to a

flavosemiquinone intermediate (FADH·) which can absorb green light. Redox potential studies imply that the flavin chromophore could exist in the semiquinone form *in vivo* and may account for the reduced sensitivity of the *hy4* mutant to green light in addition to UV-A/blue light. Transgenic tobacco plants expressing the *Arabidopsis HY4* coding sequence using the cauliflower mosaic virus 35S promoter exhibit much shorter hypocotyls in UV-A, blue and green light than those of their non-overexpressing siblings (Lin *et al.*, 1995a). Hypersensitivity to light was directly related to the level of transgene expression. The fact that overexpression of CRY1 also results in hypersensitivity to green light agrees with the conclusion that light from this region contributes to CRY1 action and may reflect the absorption properties of the bound flavosemiquinone.

Malhorta *et al.* (1995) have recently expressed CRY1, minus the C-terminal domain, as a maltose-binding protein (MBP) fusion in *E. coli*. Consistent with the findings of Lin *et al.* (1995b), the MBP-HY4 fusion had no photolyase activity. Interestingly, spectroscopic studies show that the fusion protein contains two chromophores identified as FAD and methenyltetrahydrofolate. The second chromophore is a pterin rather than the deazaflavin expected from the CRY1 amino acid sequence homolgy to other photolyases (Ahmad and Cashmore, 1993). These findings suggest that CRY1 may initiate signal transduction by a novel pathway involving electron transfer. Similarly, preliminary studies of CRY1 purified from transgenic plants overexpressing the photoreceptor also indicate the presence of a second chromophore (Lin *et al.*, 1995b). Whether or not this second chromophore is a pterin requires further investigation.

1.3.2.5 CRY1-mediated responses

In addition to the inhibition of hypocotyl elongation, CRY1 has been implicated in a number of UV-A/blue light-dependent responses. Firstly, CRY1 controls the blue light induction of anthocyanin formation by regulating the level of transcription of chalcone

synthase (CHS) and other flavonoid biosynthetic enzymes (Fig. 1.1) (Chory, 1992; Ahmad *et al.*, 1995; Jackson and Jenkins, 1995). Secondly, CRY1 is required for full expression of certain nuclear-encoded chloroplast protein genes, including ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS*) (Conley and Shih, 1995). However, the low fluence blue light-induced expression of genes encoding the LHCB1 chlorophyll-binding protein (*CAB*) is unaltered in dark-grown *hy4* seedlings (Gao and Kaufman, 1994), demonstrating that CRY1 does not regulate all blue light-regulated gene expression. More recently, Jackson and Jenkins (1995) reported that CRY1 is involved in a variety of extension-growth responses. They showed that the *hy4* mutant has longer bolted stems, increased petiole length and increased leaf width and area. Futhermore, CRY1 appears to be required for apical hook opening (Liscum and Hangarter, 1993). Taken together, these observations indicate that CRY1 is concerned with the perception of blue light in a range of extension growth and gene expression responses.

1.3.2.6 Additional cryptochrome photoreceptors

Despite extensive analysis, only one CRY1-related sequence has been identified in the *Arabidopsis* genome (Ahmad and Cashmore, 1996). This sequence, named CRY2, has extensive homology to CRY1 in the photolyase-like domain but contains a C-terminal extension of 100 amino acids with very little sequence relatedness to CRY1. Interestingly, CRY2 is almost identical to a likely UV-A/blue photoreceptor, SA-phr1, from *Sinapis alba* (Batschauer, 1993). However, SA-phr1 lacks the C-terminal extension of 100 amino acids found in CRY2. The mustard gene was isolated by screening a cDNA library using a set of oligonucleotides with degenerate sequences corresponding to a highly conserved region in the carboxy-terminal of microbial photolyases. SA-phr1, like CRY1 and CRY2, has significant sequence homology to DNA photolyases. Recently, Malhotra *et al.* (1995) expressed SA-phr1 as a MBP fusion in *E. coli* and found that it lacks photolyase activity. In fact, the *in vitro*

absorption properties resembled those of a MBP-IIY4 fusion, suggesting that SA-phr1 is a possible photoreceptor. Further studies are required to establish the precise function of SA-phr1. In contrast to CRY1, measurement of SA-*phr1* transcripts in mustard plants demonstrates that SA-phr1 gene expression is light-regulated (Batschauer, 1993). Whether CRY2 and SA-phr1 represent additional cryptochrome photoreceptors remains to be determined. As discussed below, recent biochemical and genetic approaches provide evidence for the involvement of a flavin-type UV-A/blue light receptor, unrelated to CRY1, in phototropism.

1.3.3 A photoreceptor for phototropism

1.3.3.1 Phototropic responses

Phototropism is the adaptive process by which plants bend towards a light source in order to maximise light capture for photosynthesis. In stems, phototropism is said to be positive, a bending towards the light stimulus whereas in roots, phototropism is negative, a turning away from the light stimulus. Blue and UV-A light are the most effective light qualities for inducing phototropic responses in higher plants (Iino, 1990), although green and red light-induced phototropism have been reported in some species, including *Arabidopsis* (Steinitz *et al.*, 1985). Studies have revealed a surprising complexity in the physiological bending response to blue light which can be divided into two phases depending on their fluence and time requirements (Firn, 1994). First-positive curvature is generally described as the bending towards unilateral light delivered in brief pulses at very low fluences. Second-positive curvature occurs with prolonged irradiation in a time-dependent manner. Much of the information about phototropism, as well as its puzzling complexity, has come from fluence-response measurements. Although such experiments have provided some information about the photosensor(s) mediating phototropism (e.g. Konjevic *et al.*, 1989; Konjevic *et al.*,

1992), a greater understanding has come from *Arabidopsis* mutants altered in phototropic responsiveness (Liscum and Hangarter, 1994; Liscum and Briggs, 1995).

1.3.3.2 Phototropic mutants of *Arabidopsis*

Mutants of *Arabidopsis* altered in phototropism were first described by Poff and co-workers. In total, thirty seven mutants were isolated and six of these characterised in more detail (Khurana and Poff, 1989; Khurana *et al.*, 1989). Three of the mutant lines were altered in gravitropism as well as phototropism (JK345, ZR8 and ZR19) and may be impaired in a downstream signalling event(s) common to both responses. The other three were specifically altered in phototropic-responsiveness (JK218, JK224 and JK229): strain JK218 showed no curvature to light whereas JK224 exhibited a 20-30 fold shift in the fluence threshold for blue light-dependent first-positive curvature but was unaltered in second-positive curvature (Khurana and Poff, 1989). Therefore, JK224 may be impaired in a photoreceptor pigment for first-positive phototropism. The fact that JK224 is also unaltered in green light-mediated phototropic curvature provides evidence for the existence of two separate photoreceptor systems (Konjevic *et al.* 1992). However, a recent genetic study indicates this is not the case.

Liscum and Briggs (1995) screened fast neutron-mutagenised M2 *Arabidopsis* seedlings for mutants altered in second-positive phototropism. As a result, they isolated eight further mutants altered in blue light-dependent phototropic curvature. Seven of these alleles exhibited a null mutant phenotype whereas one mutant was weakly phototropic. These *nph* (*n*on-*p*hototropic *h*ypocotyl) mutants were found to represent four genetic loci. Moreover, JK224 was found to be an allele of the *nph1* mutant series, designated *nph1-2*. Analysis of the *nph1* mutants revealed that three of the *nph1* alleles (*nph1-1*, *nph1-3* and *nph1-4*) lacked both first and second-positive phototropism in response to blue and green light. The photophysiological differences between the *nph1-2* allele (JK224) and the severe *nph1* alleles appear to indicate that both blue and green light responses are mediated by two distinct chromophores bound

to the same protein rather than two separate photoreceptors. It seems likely that the severe alleles of *nph1* remove NPH1 function, whereas the lesion in *nph1-2* affects the function of a single chromophore within the NPH1 protein, resulting in an alteration of sensitivity to blue but not green light. This is not surprising considering the null alleles were all generated by fast neutron bombardment, which often causes gross chromosomal changes such as deletions. On the other hand, JK224 (*nph1-2*) was generated by the point mutagen EMS (Khurana and Poff, 1989). Interestingly, the severe *nph1* alleles were also altered in phototropic responses to UV-A light as well as negative phototropism in roots (Liscum and Briggs, 1995). Therefore, the NPH1 protein appears to be an essential light detection or signalling component for all phototropic responses in *Arabidopsis*, including those mediated by the near UV region of the spectrum. Indeed, further evidence described below strongly supports the hypothesis that the *NPH1* locus encodes a photoreceptor for phototropism. Previously isolated phototropic response mutants, JK345, ZR8 and ZR19 were shown to be altered in gravitropism (Khurana and Poff, 1989; Khurana *et al.*, 1989). However, consistent with the expected photoreceptor function of NPH1, all of *nph1* mutants were found to be gravitropically normal (Liscum and Briggs, 1995).

1.3.3.3 The blue light-dependent phosphorylation reaction

Gallagher *et al.* (1988) reported that a brief illumination of etiolated pea stem sections reduced the phosphorylation of a 120 kDa plasma membrane protein and proposed that the high molecular weight protein substrate was an important early event in blue light signal transduction. In contrast, Short and Briggs (1990) observed that illumination of isolated pea membranes, instead of intact tissues, caused an increase in phosphorylation of the 120 kDa protein by exogenous [γ-^{32}P]ATP. Pre-illumination of the tissue with blue light before membrane isolation strongly reduced the effectiveness of the *in vitro* irradiation. Furthermore, when etiolated tissue was pre-incubated with ^{32}Pi (to allow synthesis of endogenous radiolabelled ATP), prior to illumination, an increase in

phosphorylation was detected rather than a decrease (Short *et al.*, 1992). An explanation for this phosphorylation behaviour was provided by Short *et al.* (1992). They proposed that blue light, in some manner, exposes sites in the 120 kDa protein which can be subsequently phosphorylated by endogenous ATP. Thus when tissue is irradiated *in vivo*, these sites are unavailable for phosphorylation by exogenous [γ-^{32}P]ATP. When irradiation is *in vitro*, however, endogenous ATP is no longer available and the exposed sites are rapidly phosphorylated by exogenous [γ-^{32}P]ATP. It was therefore accepted that the 120 kDa protein is phosphorylated in response to blue light. Similar blue light-induced effects have been observed in maize (Hagar *et al.*, 1993; Palmer *et al.*, 1993) and several other plant species, including *Arabidopsis* (Reymond *et al.*, 1992a). In each case, the phosphorylation response is very rapid, requiring less than 1 s illumination. Purified plasma membranes retain their phosphorylation response even when partially solubilised by detergent (Short *et al.*, 1992), demonstrating that the photoreceptor, kinase and substrate are located in the membrane fraction. Indeed, it is plausible that the photoreceptor, kinase and substrate reside on the same polypeptide or protein. Interestingly, the 120 kDa protein from pea contains a nucleotide-binding site and may have the capacity for autophosphorylation (Short *et al.*, 1993).

1.3.3.4 Blue light-dependent phosphorylation and phototropism

Short and Briggs (1990) established several photobiological correlations between the blue light-dependent phosphorylation reaction and phototropism in pea. The fluence and time dependence of the blue light-induced phosphorylation reaction are compatible with first-positive phototropism. In addition, tissue localisation of the 120 kDa protein is consistent with that predicted for the photoreceptor for first-positive phototropism. A similar correlation between the light-induced phosphorylation of a 114 kDa protein and phototropism has been made in maize (Palmer *et al.*, 1993). Compounds known to interact with flavins indicate that the photoreceptor for phototropism binds a flavin

chromophore. Similarly, Short *et al.* (1992) showed that potassium iodide (KI), sodium azide (NaN_3) and phenylacetic acid (PAA), at equivalent concentrations used to inhibit phototropism, reduce blue light-induced phosphorylation in pea membranes. Taken together, these findings provide evidence for the involvement of the blue light-dependent phosphorylation reaction in phototropism. Indeed, further studies have revealed that the 120 kDa plasma membrane protein is most likely NPH1, the putative photoreceptor for phototropism.

Reymond *et al.* (1992a) demonstrated that blue light induces the phosphorylation of a 124 kDa plasma membrane protein in *Arabidopsis*. More importantly, the phototropic mutant JK224 (*nph1-2*) was found to be defective in the blue light-dependent phosphorylation reaction (Reymond *et al.*, 1992b). Liscum and Briggs (1995) have extended this work. They found that all *nph1* mutants, including *nph1-2* (JK224), completely lacked blue light-dependent protein phosphorylation. Silver-stained SDS gels revealed that the lack of phosphorylation results from a reduced level of the 124 kDa target protein. From such gels, it was estimated that each *nph1* mutant had, at the most, only 10% of the wild-type levels of the stained protein. Therefore, the 124 kDa plasma membrane associated phosphoprotein appears to be NPH1 and probably functions as a photoreceptor for all photoropic responses in *Arabidopsis*. This hypothesis can be confirmed once the *NPH1* locus has been cloned. Further studies are also required to understand how the lesion in *nph1-2*, resulting in minor physiological changes, can dramatically reduce the level of NPH1 by 90%. Interestingly, the *nph2*, *nph3* and *nph4* mutants retain their phosphorylation activity and wild-type levels of the 124 kDa plasma membrane protein. Thus NPH2, NPH3 (*nph3-3* is an allele of JK218) and NPH4 are not required for the light-potentiated phosphorylation of the 124 kDa protein and may be downstream signal transduction components essential for the phototropic response.

1.3.3.5 NPH1 is distinct from the CRY1 photoreceptor

Several lines of evidence indicate that the NPH1 protein and the CRY1 photoreceptor are distinct. If the NPH1 protein indeed represents a flavin-type UV-A/blue light receptor then its molecular weight alone (approximately 120 kDa) distinguishes it from CRY1 and CRY2. Moreover, NPH1 is located in the plasma membrane whereas CRY1 is most likely a soluble protein. Genetic evidence has shown that phototropism is not dependent on CRY1: the *hy4* mutant exhibits wild-type levels of the 124 kDa protein and retains normal phototropic curvature in response to blue light (Liscum and Briggs, 1995). In constrast to the CRY1 photoreceptor, which mediates a range of responses (Ahmad and Cashmore, 1996), NPH1 appears to be specifically concerned with phototropism. As the *hy4* mutant is unaltered in several blue light responses, including stomatal opening and chloroplast development (Jenkins *et al.*, 1995), it is likely that additional UV-A/blue photoreceptors exist in higher plants. These may have no sequence relatedness and an entirely different chromophore composition to CRY1. The isolation of further mutants altered in particular UV-A/blue light responses will facilitate the identification of these photoreceptors and enable the cloning of the corresponding genes.

1.4 UV-B Perception

The recent discovery that contaminating man-made chlorofluorocarbons are destroying the stratospheric ozone layer has increased the importance of studying the harmful effects of UV-B (280-320 nm) radiation on living organisms. In plants, UV-B irradiation has a number of effects, including destruction of plasma membrane-associated ATPases, epidermal deformation, changes in cuticular wax composition, general growth inhibition and DNA damage (Tevini and Teramura, 1989; Stapleton, 1992). Nevertheless, plants have evolved a number of mechanisms to protect themselves against UV-B irradiation. For example, in response to potentially harmful levels of UV-B light plants can accumulate flavonoids which function as UV

protectants (Lois, 1994; Stapleton and Walbot, 1994). Flavonoids generally accumulate in the epidermis, where they are thought to prevent UV radiation from reaching underlying photosynthetic tissue (Hahlbrock and Scheel, 1989). A direct demonstration of the importance of flavonoids in protecting plants from the damaging effects of UV-B radiation has come the analysis of mutants defective in flavonoid biosynthesis. Li *et al.* (1993) studied two mutants of *Arabidopsis*, *tt4* and *tt5*, which are deficient in CHS and chalcone isomerase (CHI) respectively. As CHS and CHI catalyse the initial steps in the flavonoid biosynthetic pathway (Fig. 1.1), these mutants were unable to accumulate a range of UV-absorbing compounds normally produced in the wild-type following exposure to UV-B. The lack of production of these compounds therefore correlated with an increased sensitivity to UV-B. Similarly, Stapleton and Walbot (1994) reported that a flavonoid deficient mutant of maize has increased UV-induced DNA damage and Lois and Buchanan (1994) found that an *Arabidopsis* mutant with increased sensitivity to UV-B lacked a particular class of flavonoids. More recently, genetic analysis has implicated a role for sinapic acid esters, a class of hydroxycinnamic acid esters, in plant UV-B protection (Landry *et al.*, 1995).

Flavonoids are produced from a branch of the phenylpropanoid pathway (Fig. 1.1). Phenylalanine ammonia-lyase (PAL) is the first step in the phenylpropanoid pathway and CHS is the key point which commits the pathway to flavonoid biosynthesis. Subsequently the pathway branches to synthesise different classes of flavonoids and anthocyanins. It is well established that genes encoding key enzymes involved in flavonoid biosynthesis are induced by UV-B light (Hahlbrock and Scheel, 1989). For example, UV irradiation induces *PAL* and *CHS* gene expression in parsley cell cultures (Chappell and Hahlbrock, 1984; Hahlbrock *et al.*, 1995). In *Arabidopsis*, UV-B exposure increases transcript levels of *PAL1*, *CHS*, *CHI* and dihydroflavonol reductase (*DFR*) genes (Kubasek *et al.*, 1992; Li *et al.*, 1993). Moreover, action spectra of *PAL* and *CHS* transcript accumulation in carrot cell cultures suggest the involvement of a specific UV-B detection system (Takeda *et al.*, 1994b). It is therefore

likely that the UV-B induction of gene expression involves specific photoreception and signal transduction processes.

Although progress has been made in defining the *cis*-acting elements and transcription factors involved in the stimulation of transcription of *CHS* genes by UV-B (Hahlbrock *et al.*, 1995), very little is known about the nature of the UV-B photoreceptor(s) and the corresponding signal transduction components. With regard to UV-B perception, flavins are considered to be possible chromophore pigments (Galland and Senger, 1988a, 1988b). Support for this hypothesis comes from Khare and Guruprasad (1993) who found that flavin antagonists inhibit the UV-B induction of anthocyanin formation in maize. Furthermore, riboflavin has been shown to enhance the induction of *CHS* gene expression in response to UV-B in parsley cells (Ensminger and Schäfer, 1992). Pterins are also strong candidates for the chromophore of a UV-B photoreceptor (Galland and Senger, 1988b). Obviously, much remains to be learnt about the mechanisms by which plants perceive UV-B light. Several studies have identified a number of mutants with altered responses to UV-B (Jenkins *et al.*, 1995). However, as described above, these mutants are either altered in DNA repair or are deficient in critical enzymes required for the synthesis of UV protecting flavonoids, and therefore provide no information on UV-B perception. Hence, further genetic analysis is required to isolate additional response mutants impaired in UV-B photoreception.

1.5 Phytochrome Signal Transduction

A major improvement in our understanding of phytochrome signal transduction has come from microinjection studies and experiments with cultured soybean cells (Millar *et al.*, 1994; Bowler *et al.*, 1994b). Using the phytochrome-deficient *aurea* mutant of tomato, Chua and co-workers have developed a microinjection system for assaying the activity of introduced photoreceptor molecules, putative signalling intermediates and various pharmacological agents. Unlike the wild-type, hypocotyl cells of the *aurea* mutant fail to develop normal chloroplasts or produce anthocyanin in response to light.

However, microinjection of purified PHYA from oat into the mutant tomato cells restored chloroplast development, anthocyanin production and activation of photoregulated promoter activity equivalent to that observed in the wild-type (Neuhaus *et al.*, 1993). This rescued phenotype was only observed in *aurea* cells injected with PHYA and not in neighbouring cells, demonstrating that phytochrome signalling is cell autonomous. Neuhaus *et al.* (1993) went on to show, using various signal transduction agonists and antagonists, that G protein activation is an early signalling event. Evidence includes the observation that microinjection of the G protein activators, GTPγS and cholera toxin, induces wild-type chloroplast development and anthocyanin production in the absence of PHYA. These findings are consistent with previous reports demonstrating that heterotrimeric G proteins are involved in the phytochrome-regulated signal transduction cascade (Bossen *et al.*, 1990; Romero *et al.*, 1991b; Romero and Lam, 1993). Further microinjection experiments, and pharmacological studies using a soybean cell culture, revealed that the PHYA signalling pathway branches after the G protein into two distinct pathways which are coupled to the transcription of specific genes (Bowler *et al.*, 1994a, 1994b). One pathway is calcium/calmodulin-dependent and stimulates *CAB* and *rbcS* gene expression, whereas the other involves cGMP and induces *CHS* expression. Both pathways are required to stimulate ferredoxin $NADP^+$ oxidoreductase (*fnr*) gene expression (Fig. 1.2). In addition, these pathways exhibit reciprocal negative regulation: high activity of the calcium-dependent pathway inhibits the cGMP-dependent pathway whereas high levels of cGMP inhibit the calcium and calcium/cGMP-dependent pathways. Bowler *et al.* (1994b) proposed that the physiological significance of this type of regulation may be to prevent the production of photosynthetic components (via the calcium and cGMP-dependent pathways) in the absence of photoprotective pigments (produced by only by the cGMP-dependent pathway). Although elevations of cGMP levels in response to phytochrome have not been reported, phyochrome-induced increases in cytosolic calcium have been demonstrated (Roux *et al.*, 1986; Chae *et al.*, 1990; Shacklock *et al.*, 1992).

Protein phosphorylation has also been implicated in phytochrome signal transduction (Romero *et al.*, 1991a; Bowler *et al.*, 1994b; Hamada and Hasunuma, 1994). Interestingly, Schneider-Poetsch (1992) observed sequence homology between the C-terminal region of phytochrome polypeptides and the histidine kinase domain of a family of bacterial sensory proteins. Similarly, a novel phytochrome from the moss *Ceratodon purpureus* exhibits striking sequence homology to the catalytic domain of eukaryotic protein kinases (Algarra *et al.*, 1993). Therefore, the light activation of phytochrome may be directly linked to a phosphorylation event.

Our understanding of phytochrome signal transduction has revealed that signalling mechanisms identified in animal cells are also operative in plants. However, not all animal signalling systems are directly applicable to plant cells. For example cAMP, an important molecule in animal signalling, is much less significant in plants (Trewavas and Gilroy, 1991; Bolwell, 1995). In some cases, plant signalling appears more analogous to bacterial signalling systems (Hughes, 1994), whereas in others it may be altogether novel (Deng *et al.*, 1992; Terzaghi and Cashmore, 1995b). Despite the considerable progress made by Chua and co-workers, there are still many questions concerning phytochrome signalling to be addressed. For instance, G protein activation is the earliest event in the proposed PHYA pathway, but phytochrome is localised in the cytosol and heterotrimeric G proteins are normally coupled to transmembrane receptors. This suggests the requirement for additional, perhaps novel, components to couple phytochrome photoreception to G protein activation. Whether the cGMP and calcium pathways identified are used specifically by PHYA or are shared by different phytochromes is another interesting question. Although these pathways have been linked to PHYA (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994a), it is also possible that they are used by the other phytochromes or even UV/blue photoreceptors.

1.6 UV-A/blue Light Signal Transduction

In contrast to phytochrome signalling, our knowledge of UV-A/blue light signal transduction is far less advanced. Although some progress has been made in identifying likely signalling events associated with UV-A/blue photoreception, further work is required to relate these events to specific photoreceptors and particular downstream responses, especially gene expression (Kaufman, 1993; Jenkins *et al.*, 1995). Perhaps the best approach has been to search for rapid phenomena resulting from a brief illumination with UV-A/blue light. As described below, such studies have mainly employed the use of standard biochemical and cell physiological techniques.

1.6.1 Membrane potentials and the proton-ATPase

Several electrophysiological techniques have been used to identify UV-A/blue light-mediated effects on plant membranes. For example, Spalding and Cosgrove (1989) discovered, by means of microelectrode impalement, a large transient blue light-induced depolarisation of the plasma membrane in cucumber hypocotyls. They noted that the membrane depolarisation preceded the blue light inhibition of hypocotyl elongation and suggested the change in potential may reflect a signal transduction step associated with this growth response.

Spalding and Cosgrove (1992) went on to examine the effects of various ion channel inhibitors on this response. Vacuum infiltration was used to introduce ion channel blockers into the extracellular spaces of cucumber hypocotyl segments. They found that vanadate, an inhibitor of the plasma membrane proton-ATPase, significantly reduced the depolarisation event. KCN, which can inhibit the proton-ATPase by blocking ATP production, was also effective. The proton-ATPase is an important factor controlling the polarized state of the plant plasma membrane (Spanswick, 1981) and the authors proposed that blue light could mediate a membrane depolarization by inhibiting this electrogenic component. Support for this hypothesis came from the observation that blue light irradiation caused a rapid increase in ATP levels. A role for

calcium in the repolarisation mechanism was also implicated. However, inhibition of the proton pump was calculated to be insufficient to account for the full extent of depolarisation and it was suggested that the process also involves the activation of anion channnels. Indeed, blue light-induced depolarisation has recently been shown to involve the activation of a plasma membrane anion channel in *Arabidopsis* hypocotyl cells (Cho and Spalding, 1996). The blue light-induced activation of the anion channel was potently and reversibly blocked by the anion channel blocker 5-nitro-2,3-phenylpropylaminobenzoic acid (NPPB). In addition, NPPB blocked the blue light depolarisation *in vivo* and decreased the effect of blue light on hypocotyl elongation. The authors therefore proposed that anion channel activation plays a role in transducing blue light to growth inhibition.

Similar blue light-induced depolarisation events have been observed in *Phaseolus* pulvinar motor cells (Nishizaki, 1988). This membrane potential change is thought to be associated with blue light-regulated leaf movement and may also involve inactivation of the proton-ATPase (Nishizaki, 1994). In contrast, blue light appears to activate the plasma membrane proton-ATPase in stomatal guard cells. During stomatal opening, proton extrusion by guard cells (Shimazaki *et al.*, 1986) creates an electrochemical gradient (Assmann *et al.*, 1985) that drives K^+ and Cl^- uptake. K^+ and Cl^- entry causes a rise in osmotic potential, resulting in water influx, a swelling of the guard cells and consequent stomatal opening. An involvement of the proton-ATPase in blue light-stimulated proton extrusion has been demonstrated by the inhibitory effects of vanadate (Gepstein *et al.*, 1982; Schwartz *et al.*, 1991; Amodeo *et al.*, 1992). Moreover, fusicoccin, an activator of the proton-ATPase (Johansson *et al.*, 1993), induces swelling of *Vicia* guard cell protoplasts (Assman and Schwartz, 1992; Shimazaki *et al.*, 1993; Goh *et al.*, 1995). However, conflicting results have been reported regarding the effectiveness of vanadate in the guard cell system. Shimazaki *et al.* (1986) detected no inhibition by vanadate of both proton extrusion and stomatal opening in *Vicia* guard cells. An explanation for this discrepancy may be due to problems in vanadate uptake (Schwartz *et al.*, 1991). While the above findings strongly suggest that blue light

regulates the activity of the plasma membrane proton-ATPase, the mechanism(s) involved is yet to be determined.

1.6.2 Redox reactions and electron transport

Oxidation-reduction (redox) reactions occur when electrons are transferred from donor molecules to acceptors. Much attention has focused on mitochondria, chloroplasts and the endoplasmic reticulum, well known sites of redox activity. However, electron transport also occurs at the plasma membrane of both plant and animal cells (Rubinstein and Luster, 1993). For some time now, it has been suggested that a blue light-activated plasma membrane redox system, distinct from the proton-ATPase, is responsible for proton pumping by stomatal guard cells (Raghavendra, 1990). Evidence for this hypothesis has come from studies using protonophores and inhibitors of redox processes. Vani and Raghavendra (1989) identified, by the reduction of tetrazolium salts, a blue light-induced redox activity at the plasma membrane of *Vicia* stomatal guard cells. This redox system requires NADH and is insensitive to KCN. Similarly, redox activity of *Pisum* guard cell protoplasts is unaffected by vanadate and fusicoccin (Vani and Raghavendra, 1992). In each case, the insensitivity to ATPase inhibitors or fussicoccin indicates that the guard cell redox activity does not involve the plasma membrane proton pump. More importantly, Gautier *et al.* (1992) reported that incubation of *Commelina* guard cell protoplasts with the electron acceptor, ferricyanide, reduced proton efflux in response to blue light. Proton pumping was accompanied by oxygen uptake and the authors proposed that oxygen functions as the terminal electron acceptor of the redox chain. However, it is suggested that the plasma membrane redox system is not the only driving force of the proton efflux required for K^+ uptake during stomatal opening (Vani and Raghavendra, 1992). Thus further research is needed to establish the relative contributions of the redox system and the proton-ATPase to proton efflux.

Blue light-stimulated plasma membrane electron transport has also been observed in *Avena* mesophyll cells (Dharmawardhane *et al.*, 1989). Plasma membrane redox activity was studied by measuring the reduction of exogenous ferricyanide. Interestingly, the effect of blue light on electron transfer could only be detected in leaf segments pretreated with sphingoid bases, potent inhibitors of protein kinase C. Thus de-phosphorylation of a redox protein(s) may be essential for the stimulation of electron transport in *Avena* mesophyll cells.

Further evidence suggests a plasma membrane redox activity is involved in the suppression of hypocotyl extension. The reducing agent, ascorbate, was found to alleviate the blue light-induced inhibition of cucumber hypocotyl elongation (Shinkle and Jones, 1988). Ascorbate also inhibited cell wall peroxidase activity purified from the cucumber hypocotyl segments and it was proposed that blue light stimulates activation of the peroxidase via oxidation-reduction reactions. More recently, ascorbate, in addition to other reducing compounds, has been shown to enhance the blue light-induced phosphorylation of a 100 kDa protein in plasma membranes isolated from maize coleoptiles (Hagar *et al.*, 1993). The properties of the maize protein are similar to the putative photoreceptor for phototropism identified in a number of plant species (Reymond *et al.*, 1992a; Liscum and Briggs, 1995). The reducing agents may therefore facilitate the transfer of electrons closely associated with blue light photoreception and/or protein phosphorylation.

Taken together, the above findings strongly suggest that electron transfer is an important signalling event following UV-A/blue light perception in plants. Since CRY1 contains a flavin chromophore (Lin *et al.*, 1995b; Malhotra *et al.*, 1995) it is possible that redox reactions and electron transport are early steps in CRY1 signal transduction.

1.6.3 G proteins

It is well established that GTP-binding proteins exist in higher plants (Terryn *et al.*, 1993; Kaufman, 1994). While G protein activation is an early event in the

phytochrome signalling pathways (Bossen *et al.*, 1990; Romero *et al.*, 1991b; Neuhaus *et al.*, 1993), separate studies provide evidence for G protein involvement in blue light signal transduction. Warpeha *et al.* (1991) reported that blue light stimulates a transient increase of GTPase activity in plasma membrane-enriched fractions isolated from etiolated pea apices. Only a brief illumination (15 s) and a low fluence of blue light was required for this rapid response whereas red light had no effect. Non-hydrolysable competitve inhibitors of heterotrimeric G proteins, GDPβS and imidodiphosphate (GppNHp), completely abolished the blue light-mediated GTPase activity. Blue light did not induce [γ-^{32}P]ATP hydrolysis in this assay, indicating that the reaction was specific to GTP. Moreover, blue light irradiation stimulated binding of the radiolabelled GTP analogue, GTP[γ^{35}S] to the pea plasma membrane fraction. Warpeha and co-workers then went on to identify a 40 kDa plasma membrane protein using antibodies raised against mammalian G protein α-subunits, including transducin-α. The 40 kDa polypeptide identified was ADP-ribosylated by pertussis and cholera toxins, a feature exhibited by particular mammalian G proteins (Kaufman, 1994). Since blue light promoted binding of a GTP photoaffinity label to a polypeptide of similar size, it seems likely that the 40 kDa protein is a α-subunit of a typical heterotrimeric G protein involved in a blue light signalling pathway of an unidentified low fluence response. Interestingly, the fluence threshhold for blue light excitation of the G protein resembles that for the blue light induction of *CAB* gene expression in pea (Gao and Kaufman, 1994). Further studies indicate the photoreceptor involved in the blue light-induced GTPase activity is likely to be a flavoprotein (Warpeha *et al.*, 1992). The flavin antagonists, PAA and KI, inhibit the ability of blue light to stimulate GTPγS binding to the pea membrane protein. Although these compounds may also affect pterins, fluorescence emission data indicate the presence of flavins in the pea plasma membrane fraction.

1.6.4 Calcium

Calcium is known to function as an important second messenger in a variety of plant responses (Bush, 1993). However, the evidence for calcium involvement in UV-A/blue light signal transduction is rather limited. Probably the best example is the observation by Russ *et al.* (1991) that UV-A and to a lesser extent blue light, stimulate an increase in cytosolic calcium in the green algae *Mougeotia*. The authors proposed that the increase in cytosolic calcium is involved in light-regulated chloroplast movement. Several other studies, involving the use of calcium chelators and calcium channel blockers, provide indirect evidence for a requirement for calcium in UV-A/blue light signalling. For instance, Shinkle and Jones (1988) demonstrated, using the calcium chelator EGTA, that the blue light inhibition of hypocotyl elongation in cucumber seedlings requires extracellular calcium. As the cucumber seedlings were previously grown in red light, the response is likely to be specific to blue light. Irradiation of rose cells by UV light stimulates a rapid and specific efflux of K^+ (Murphy and Wilson, 1982). Pre-treatment of rose cells with EGTA inhibits the UV-stimulated K^+ efflux (Murphy, 1988). Similarly, the K^+ efflux is also inhibited by lanthanum, which competes externally with calcium for plasma membrane calcium channels (Tester, 1990). These findings therefore indicate a role for extracellular calcium in this UV-mediated response.

1.6.5 Calmodulin and calcium-dependent kinases

A role for calmodulin has been implicated in the blue light-stimulated proton efflux from *Vicia* guard cell protoplasts. Shimazaki *et al.* (1992) reported that blue light-dependent proton pumping is inhibited by a number of calmodulin antagonists, including W-7. Inhibitors of the mammalian calcium/calmodulin-dependent myosin light chain kinase (MLCK), ML-7 and ML-9, were also effective whereas only a small reduction was observed with inhibitors of protein kinase C. No effects were detected with inhibitors of cyclic nucleotide-dependent protein kinases or calmodulin-dependent

protein kinase II. In accordance with these results, light-induced stomatal opening in *Commelina* epidermal strips is also inhibited by W-7 and ML-9. Therefore, a MLCK-like protein may be involved in the signal transduction pathway coupling blue light photoreception to proton efflux in stomatal guard cells. Shimazaki *et al.* (1993) later demonstrated that fusicoccin restores proton pumping and stomatal opening previously inhibited by W-7 and ML-7. Thus the inhibitory action of these compounds is specific to the light signalling pathway and not due to a direct effect on the proton pump. In contrast, PMA, CCCP and DCCD, inhibitors of the plasma membrane proton-ATPase, suppressed proton extrusion and stomatal opening. Fusicoccin was unable to restore stomatal responses in the presence of these inhibitors. This is consistent with the conclusion that a MLCK-like protein is involved in the blue light response of stomata.

1.6.6 Inositol phospholipid metabolism

The inositol phospholipid pathway, an important aspect of animal signalling, is also operative in plants but has proved difficult to characterise (Trewavas and Gilroy, 1991). This pathway involves the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C in the plasma membrane to release the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). In animal systems, DAG activates protein kinase C whereas IP_3 releases calcium from intracellular stores thus activating calcium-dependent enzymes and altering cell physiology. While components of the inositol phospholipid pathway are present in plant cells, evidence suggests that there are significant differences between the plant and animal phosphoinositide systems (Drøbak, 1992). For example, it was originally considered that no functional equivalent for protein kinase C exists in plants. However, protein kinase C has recently been purified and characterised from *Brassica campestris* (Nanmori *et al.*, 1994).

Light-induced phospholipid turnover has been observed in *Samanea* pulvini (Morse *et al.*, 1987). Only a brief irradiation of excised pulvini with white light (15-30

s) is required to decrease PIP_2 levels and increase the levels of IP_3 and DAG. Although Morse *et al.* (1989) proposed that a blue light-absorbing pigment was involved in this light-stimulated reaction, no evidence for this has been published. Indeed, the light-stimulated phospholipid turnover may be due to the activation of phytochrome. Phytochrome has been shown to control phospholipid turnover in maize (Guron *et al.*, 1992). Nevertheless, Kim *et al.* (1996) have recently shown that the generation of IP_3 is an early event in the signal transduction pathway by which blue light closes K^+ channels in in *Samanea* pulvinar cells.

1.7 UV-B Signal Transduction

Very little information is available regarding UV-B signal transduction in plants. Perhaps the best attempt to elucidate UV-B signal transduction has come from a recent study on the pathogenesis-related protein PR-1 from tobacco. Green and Fluhr (1995) reported that UV-B induces PR-1 accumulation in tobacco leaves. The PR-1 induction by UV-B was preceded by an increase in *PR-1* transcripts. Pre-treatment of tobacco leaves with cycloheximide inhibited *PR-1* transcript accumulation, indicating a requirement for cytoplasmic protein synthesis. In addition, the antioxidants *N*-acetyl-L-cysteine and pyrolidine dithiocarbamate inhibited UV-B-induced PR-1 accumulation whereas rose bengal, a generator of active oxygen, caused an accumulation of PR-1 in the absence of UV-B. The authors therefore proposed that the signalling pathway coupling UV-B perception to PR-1 accumulation involves protein synthesis and the generation of reactive oxygen species (ROS). Interestingly, UV-C irradiation has been shown to increase levels of ROS in rose cells (Murphy and Huerta, 1990). UV-induced defense responses in animal cells have also been reported to involve ROS (Devary *et al.*, 1992). Hence similar defense mechanisms in response to UV light may exist between plant and animal systems. Nevertheless, it is premature to conclude that all gene expression responses induced by UV-B are mediated by ROS.

1.8 Mutants Defining Downstream Signalling Components

1.8.1 *Arabidopsis* 'de-etiolated' mutants

Further photomorphogenic mutants have been isolated which appear to affect signal transduction events subsequent to photoreception. For example, eleven loci have recently been defined by a series of *Arabidopsis* mutants that aberrantly undergo photomorphogenesis in the dark. Mutants of this phenotype have been called de-etiolated (*det*) (Chory *et al.*, 1989b; Chory, 1993) and constitutive photomorphogenic (*cop*) (Deng *et al.*, 1992; Deng, 1994). When grown in complete darkness, *det/cop* mutants develop a number of features normally associated with light-grown plants, including cotyledon expansion, suppression of hypocotyl extension, the expression of light-regulated genes such as *CAB* and *CHS* and increased anthocyanin formation. In the light, adult plants are often dwarf and have reduced fertility. A similar mutant to *det1* and *cop1* has recently been reported in pea, which exhibits light-independent photomorphogenesis (*lip*) (Frances *et al.*, 1992). The phenotypic characteristics of the *det/cop* mutants differ depending on the locus and the allele. Among these, the dark-grown *det1*, *cop1*, *cop8*, *cop9*, *cop10* and *cop 11* mutants exhibit the most pleiotropic phenotypes.

The interpretation of these recessive mutants is that the corresponding wild-type gene products function as negative regulators of gene expression and photomorphogenic development in the dark. According to this hypothesis, light perceived by specific phororeceptors will act to remove the negative regulatory activity. Support for this hypothesis comes from the recent finding that overexpression of wild-type COP1 in transgenic *Arabidopsis* partially suppresses photomorphogenic development in light-grown plants (McNellis *et al.*, 1994). Double mutants have been produced between several *det/cop* mutants and phytochrome-deficient or CRY1-deficient mutants (Chory, 1992; Ang and Deng, 1994; Miséra *et al.*, 1994; Wei *et al.*, 1994a, 1994b). In each case, the double mutants resemble the *det/cop* parent, indicating that the DET/COP components function downstream of phytochromes and

CRY1. These data therefore imply that the DET and COP gene products occupy a central position in a light signalling network, acting as master switches controlling the transition from etiolation in darkness to normal development in the light.

In addition to the characteristics described above, *det/cop* mutants are altered in the tissue-specific regulation of gene expression. For instance, plastid development and *CAB* gene expression occurs in the roots of *det1* and *cop1* mutants (Chory and Peto, 1990; Deng and Quail, 1992). Moreover, Chory and Peto (1990) observed that *CHS* promoter activity in *det1* seedlings is no longer restricted to the epidermis of the plant but expressed in all leaf cell types. These observations demonstrate that certain DET and COP gene products mediate the effects of spatial regulatory signals as well as transducing multiple light signals.

1.8.2 *Arabidopsis fusca* mutants

Several of the *det* and *cop* mutants have been isolated in earlier mutant screens on the basis of their aberrant phenotype in embryos and light-grown seedlings. For example, *det1*, *cop1* and *cop9* mutants proved to be weak alleles of *fusca* mutants, *fus2*, *fus1* and *fus7* respectively (Miséra *et al.*, 1994). The *fusca* (from the latin 'dark purple') mutants represent strong alleles and are characterised by the synthesis of high levels of anthocyanin in immature embryos and seedlings and by adult lethality (Castle and Meinke, 1994; Miséra *et al.*, 1994). Adult lethality is not due the overproduction of anthocyanin as double mutants produced between *fusca* alleles and mutants defective in anthocyanin biosynthesis still exhibit abnormal development in the absence of anthocyanin (Castle and Meinke, 1994; Miséra *et al.*, 1994). The severe nature of the *fusca* phenotype demonstrates that the corresponding gene products are essential for normal growth and development. There is evidence that the DET/COP/FUS gene products are involved in the transduction of a range of environmental and endogenous signals, since they show either constitutive or hyper-responses to a range of metabolites and growth regulators (Castle and Meinke, 1994).

1.8.3 Characterisation of DET/COP proteins

The *COP1*, *COP9*, *COP11* and *DET1* loci have been cloned (Deng, 1994). Although *COP9*, *COP11* and *DET1* have no sequence homology with other proteins, the *COP1* locus encodes a 74 kDa protein which contains a number of recognisable structural motifs: a zinc-finger domain at the N-terminus, a putative coiled-coil region and a domain at the C-terminus with multiple WD-40 repeats similar to those in the β-subunit of heterotrimeric G proteins (Deng, 1992). COP1 also shows significant sequence homology with the $TAF_{II}80$ subunit of the *Drosophila* transcription factor $TF_{II}D$ in all but the zinc-binding domain. Thus COP1 may interact with DNA directly as well as with other components of the light signalling network. Consistent with the conclusion that COP1 acts as a transcriptional regulator is the finding that both COP1 and DET1 sequences have functional nuclear localisation signals. Indeed, the nuclear localisation of COP1 and DET1 has recently been confirmed by expressing COP1 and DET1 as β-glucuronidase (GUS) fusion proteins (von Arnim and Deng, 1994; Pepper *et al.*, 1994). In contrast, the COP9 and COP11 proteins show no indication of nuclear localisation. However, both appear to be hydrophilic and contain several potential sites for phosphorylation (Wei *et al.*, 1994a, 1994b).

1.8.4 COP1 localisation is regulated by light

Further research has been directed towards understanding how COP1 is inactivated by light. Studies by von Armin and Deng (1994) indicate that COP1 is in fact a nuclear protein whose intracellular localisation is regulated by light. They introduced constructs designed to express GUS-COP1 fusion proteins into the epidermal cells of onion bulbs by particle bombardment. The intracellular localisation of these proteins were then determined by histochemical staining for GUS activity in cells kept either in the light or in the dark. Approximately 25% of the transformed cells kept in the dark showed nuclear enrichment of the fusion protein, whereas GUS-COP1 was exclusively located in the cytosol in light-treated cells. Hence these results provide a potential

mechanism for the inactivation of COP1 which involves the light-dependent movement of COP1 out of the nucleus and into the cytosol. Similar light-dependent COP1 movements were observed with *Arabidopsis* plants stably transformed with the fusion construct expressing GUS-COP1 (von Arnim and Deng, 1994). In *Arabidopsis* hypocotyl cells, the nuclear levels of GUS-COP1 changed in response to dark/light transitions: the GUS-COP1 fusion protein became localised in the cytosol when dark-grown plants were transferred to the light and became nuclear-localised when light-grown plants were transferred to the dark. The light-mediated migration of GUS-COP1 out of the nucleus took almost 12 hours to be detected and reached a maximum after 36 hours. Since many light-regulated developmental changes, such as the inhibition of hypocotyl elongation, occur within minutes, these observations make it difficult to envisage COP1 functioning as a direct repressor of photomorphogenesis. However, von Arnim and Deng proposed that the slow kinetics for GUS-COP1 movement are due to properties of the fusion protein. The GUS-COP1 fusion is a larger protein than the endogenous COP1 protein and not native to *Arabidopsis* cells. Therefore, it is likely that the kinetics for GUS-COP1 subcellular trafficking will be different from the endogenous COP1 protein. Furthermore, the fact that the GUS-COP1 fusion can restore *cop1* mutations to the wild-type phenotype demonstrates that GUS-COP1 can substitute for the endogenous COP1 protein (von Arnim and Deng, 1994). Alternatively, it is possible that the translocation of COP1 to the cytosol results from the light-induced inactivation of COP1: COP1 might first be inactivated and then diffuse out of the nucleus after dissociating from potential targets. If this is the case, further studies are required to determine how light inactivates COP1.

von Arnim and Deng (1994) also observed that the GUS-COP1 fusion remained localised in the nucleus of root cells even in light-grown *Arabidopsis* plants. As root cells do not undergo photomorphogenesis, these findings are consistent with the role of COP1 as a repressor of photomorphogenic development. Furthermore, preliminary immunocytological data involving protoplasts isolated from light and dark-grown cells indicate that endogenous COP1 has a similar intracellular distribution to that of the

GUS-COP1 (von Armin and Deng, 1994). More recently, Matsui *et al.* (1995) have isolated a protein, COP1-interactive protein 1 (CIP1), which interacts with the coiled-coil region of COP1 *in vitro*. Immunofluorecent labelling of CIP1 in protoplasts isolated from *Arabidopsis* seedlings indicates that CIP1 is associated with the cytoskeleton in hypocotyl and cotyledon cells but not in roots. Therefore, CIP1 may be involved in regulating the light-dependent and cell-specific intracellular location of COP1.

1.8.5 COP9 is a component of a novel cytosolic complex

Recent biochemical evidence demonstrates that several of the COP proteins are likely to be components of a novel signalling complex situated in the cytosol. *COP9* encodes a 22.5 kDa polypeptide which is present in large complexes of varying sizes in dark-grown *Arabidopsis* seedlings (Wei *et al.*, 1994a). A more homogeneous COP9 containing complex of approximately 560 kDa was obtained when seedlings were exposed to light. This large complex requires COP8 and COP11 for its formation or stability because it is absent in *cop8* and *cop11* mutants. On the other hand, formation of the complex was unaffected by mutations in *cop1*, *cop10* and *det1* loci. Therefore, these findings suggest that COP9 is part of a large complex whose organisation is influenced by light and requires the presence of COP8 and COP11. Moreover, they provide evidence for molecular interactions between different *COP* gene products. Clearly further studies are required to determine the nature of these interactions.

1.8.6 *Arabidopsis icx1* mutant

Mutants which appear to have an enhanced sensitivity to light have also been reported. For example, the high pigment mutant of tomato (*hp*) shows an increased phytochrome-induction of anthocyanin formation in the absence of blue light (Peters *et al.*, 1989). It is proposed that the *hp* mutant may be altered in a signalling component

linking phytochrome and blue light signal transduction. More recently, Jackson *et al.* (1995) have identified a mutant altered in anthocyanin formation and *CHS* expression. This mutant, termed *icx1* (*i*ncreased *c*halcone synthase expression), was isolated by screening mutagenised transgenic seedlings for altered expression of GUS fused to the promoter of the *Sinapis alba* (white mustard) *CHS-1* gene. The *icx1* mutant has elevated GUS activity over a range of fluence rates of white light compared to the transgenic wild-type. Increased GUS activity was found to correspond with an increase in *CHS* transcript levels. Moreover, *CHI* and *DFR* transcript levels were also elevated as was anthocyanin production, whereas the phenotype of dark-grown *icx1* seedlings appears to be normal. Therefore, the *icx1* mutant appears to have increased sensitivity to light with respect to light-regulated expression of genes involved in flavonoid biosynthesis. An interpretation of this recessive mutation is that *ICX1* encodes a negative regulator which normally constrains the expression of *CHS* and other genes in the light. Interestingly, no alteration in *CAB* transcript levels was observed, indicating that *icx1* is not altered in the expression of all light-regulated genes. The *icx1* mutant is also altered in several aspects of epidermal development, including a reduced level of leaf trichomes and alterations to the seed coat. Indeed, *icx1* is altered in a similar range of processes to the *ttg* (*transparent testa, glabra*) mutant, which is deficient in trichomes, seed mucilage and anthocyanin synthesis (Koornneef *et al.*, 1981). As a consequence, the seed testa of *ttg* is unpigmented. On the basis of these results it is likely that ICX1 acts in the epidermis to control aspects of gene expression and development.

1.9 Interactions Between Photomorphogenic Loci

Isolation of the *det/cop* mutants and subsequent cloning of some of these loci have provided important insights into the complex signalling network associated with photomorphogenic development. The *hy* mutants provide evidence that there are specific red/far-red and UV-A/blue photoreceptors and that red/far-red and UV-A/blue

light signal transduction pathways are genetically separable (Koornneef *et al.*, 1980; Liscum and Hangarter, 1991). Since photomorphogenesis requires the concerted action of multiple photoreceptors it seems likely that these individual pathways converge downstream upon common regulatory elements. The pleiotropic phenotypes caused by the *COP1*, *COP8*, *COP9*, *COP10*, *COP11* and *DET1* loci imply that these genes encode such common regulatory elements. Indeed, genetic studies, involving double mutants, demonstrate that *det* and *cop* mutations are epistatic to phytochrome-deficient and CRY1-deficient mutants (Chory, 1992; Chory, 1993; Ang and Deng, 1994; Wei *et al.*, 1994a, 1994b). The large cytosolic complex identified by Wei *et al.* (1994a) may occupy a central position in the signalling network contolling the light regulation of seedling development. COP1 and DET1 are likely to be closely associated with the complex as mutations in these loci are also pleiotropic. In turn, such common components may function as master switches, transmitting information to various cellular effectors via a number of branched signalling pathways.

The *det3* mutant and to some extent *cop2* and *cop3* mutants have a de-etiolated morphology in the dark but exhibit a wild-type pattern of light-regulated gene expression (Cabrera y Poch *et al.*, 1993; Hou *et al.*, 1993). Thus DET3, COP2, and COP3 are possible regulatory components defining downstream branches in the light-regulated signal transduction pathway. Similarly, Li *et al.* (1994) have isolated several *Arabidopsis* mutants which are specifically altered in one particular aspect of light-regulated development. These so called *doc* mutants (*d*ark *o*verexpression of *C*AB) have normal etiolated morphology in the dark but exhibit aberrant *CAB* gene expression. Epistatis studies indicate that these new genes define downstream branches of the light signal transduction pathway that are specifically concerned with the regulation of *CAB* gene expression. Further genetic analysis is beginning to establish models outlining the regulatory hierarchy between photoreceptors and the different downstream signalling components. The evidence places DET1 and HY5 upstream of COP1, COP8, COP9, COP10, and COP11, with a possible direct interaction between COP1 and HY5 (Ang and Deng, 1994). Although a genetic dissection of the light-

signalling network is well underway, it will be important to determine the biochemical mechanisms coupling photoreception to the COP-DET-FUS components. Obviously, the phytochrome signal transduction components identified by Chua and co-workers, such as G proteins, calcium/calmodulin and cGMP, are likely candidates.

1.10 Conclusions

In recent years considerable progress has been made in understanding the mechanisms involved in light-regulated plant development. A combination of biochemical and genetic approaches have unveiled important insights into plant photosensory perception and signal transduction. Like the phytochromes, several UV/blue photoreceptors appear to exist in plants. Moreover, it is now apparent that light signals perceived by multiple photoreceptors are channelled through a master processing centre which mediates the processes associated with photomophogenic development via a number of branched signalling pathways. The balance of flux through these pathways will ultimately determine the specific cellular response. Although primary phytochrome signal transduction events have been identified, much less is known about the upstream signalling events following UV/blue photoreception. It is therefore essential to develop systems in which the mechanisms coupling UV/blue light perception to defined downstream responses, in particular gene expression, can be dissected. The isolation of further UV/blue response mutants will facilitate the identification of these upstream components and their corresponding photoreceptors.

1.11 Aims of this Study

The overall aim of this study was to investigate signal transduction mechanisms concerned with the UV/blue light regulation of gene expression. The *CHS* gene of *Arabidopsis* was chosen as a system to study UV/blue light signal transduction for several reasons. Firstly, similar to parsley, *CHS* is encoded by a single gene in

Arabidopsis (Feinbaum and Ausubel, 1988) and therefore presents a system of reduced complexity in comparison to other species, such as bean, which contains approximately eight *CHS* genes (Hahlbrock and Scheel, 1989). Secondly, the photoregulation of *CHS* expression in *Arabidopsis* has been well characterised. In contrast to several other species, phytochrome appears to have very little involvement in the regulation of *CHS* in *Arabidopsis* (Kaiser *et al.*, 1995; Batschauer *et al.*, 1996), whereas expression is specifically induced by UV-B and UV-A/blue light (Kubasek *et al.*, 1992; Li *et al.*, 1993; Jackson *et al.*, 1995). Hence stimulation of the *Arabidopsis CHS* gene by UV-B and UV-A/blue light provides an ideal system for investigation of the signalling processes involved. Another important feature of *Arabidopsis* is that a number of mutants have been isolated which are altered in the ability to respond to UV-A/blue light (Jenkins *et al.*, 1995). These mutants may therefore facilitate the identification of signal transduction events associated with UV/blue light perception.

The initial aim of this project was to establish a suitable system that would permit biochemical experimentation. We chose to use an *Arabidopsis* cell suspension culture first introduced by May and Lever (1993). This approach is similar to that taken with the soybean cell culture, which has provided information on the phytochrome signalling pathway regulating *CHS* (Lam *et al.*, 1989a; Romero and Lam, 1993; Bowler *et al.*, 1994a, 1994b). The *Arabidopsis* cell culture was therefore used to address the following questions: (i) What are the components of the UV-B and UV-A/blue light signalling pathways regulating *CHS* expression? (ii) Are the UV-B and UV-A/blue phototransduction pathways distinct and are any of the intermediates shared? (iii) Are the UV-B and UV-A/blue phototransduction pathways distinct from the phytochrome signalling pathway regulating *CHS* in other species? (iv) Do similar UV/blue light signalling pathways regulate other flavonoid biosynthesis genes? (v) What are the *cis*-acting regulatory elements concerned with the transcriptional activation of *CHS* by UV-B and UV-A/blue light? (vi) Can such an approach be extended to *Arabidopsis* mutants?

Figure 1.1. A simplified representation of the phenylpropanoid and flavonoid biosynthesis pathway

The reactions catalysed by the enzymes phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI) and dihydroflavonol reductase (DFR) are illustrated. The boxed steps represent the core reactions of general phenylpropanoid metabolism. PAL catalyses the inital step of these core reactions. The subsequent reactions of general phenylpropanoid metabolism are catalysed by C4H and 4CL. CHS is the first committed enzyme of flavonoid biosynthesis. DFR is the first committed enzyme of anthocyanin biosynthesis.

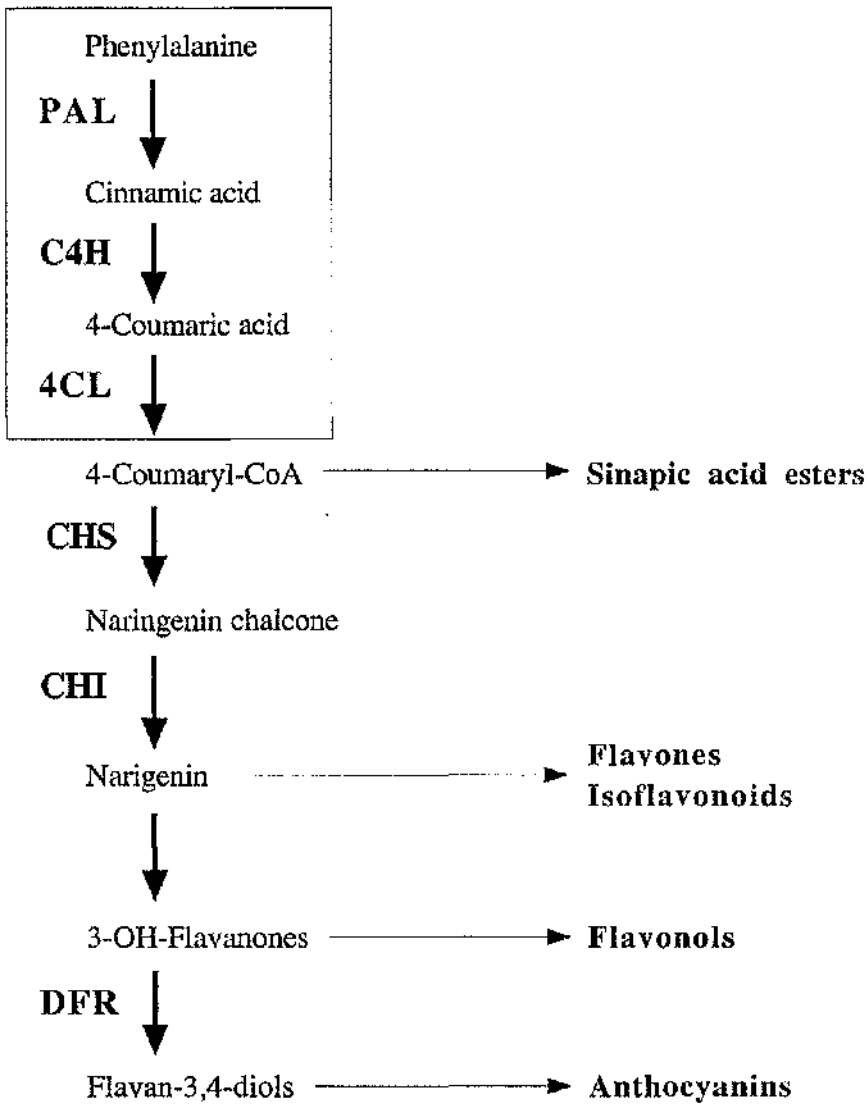
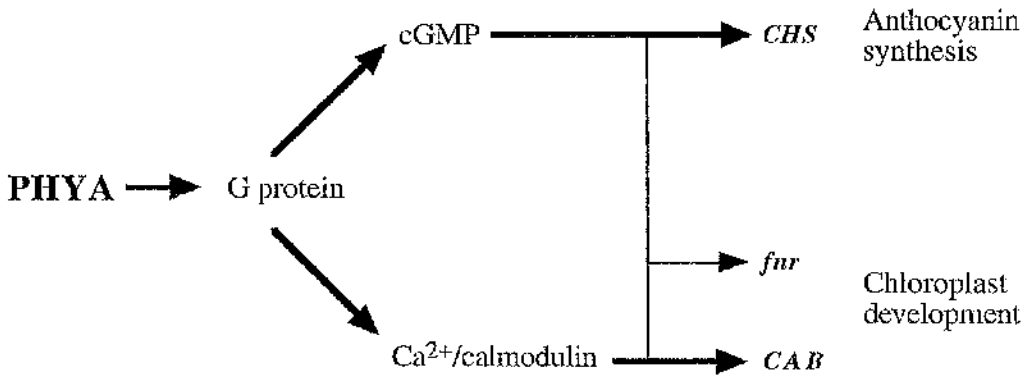

Phenylalanine
PAL
Cinnamic acid
C4H
4-Coumaric acid
4CL
4-Coumaryl-CoA
Sinapic acid esters
CHS
Naringenin chalcone
CHI
Narigenin
Flavones
Isoflavonoids
3-OH-Flavanones
Flavonols
DFR
Flavan-3,4-diols
Anthocyanins

Figure 1.2. Proposed model of the phytochrome A signal transduction pathways

The model shown was deduced from microinjection experiments with the phytochrome-deficient *aurea* mutant of tomato and pharmacological studies using a photomixotrophic soybean cell culture (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994a, 1994b). Phytochrome is proposed to activate one or more heterotrimeric G proteins, which in turn activates two parallel signalling pathways: a Ca^{2+}/calmodulin-dependent pathway and another involving cGMP. The cGMP pathway is responsible for *CHS* gene expression and anthocyanin biosynthesis whereas the Ca^{2+}/calmodulin pathway is required for the synthesis of components for chloroplast development, including *CAB* gene expression. Both pathways are required for *fnr* gene expression and full chloroplast development.


PHYA
G protein
cGMP
Ca2+/calmodulin
CHS
fnr
CAB
Anthocyanin synthesis
Chloroplast development

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The chemicals used in this study were obtained from BDH (AnalaR® grade, Poole, Dorset) unless otherwise indicated.

2.1.2 Radiochemicals

[α-^{32}P]dCTP was supplied by Amersham International (UK) and [^{3}H]chloramphenicol by NEN® (DuPont Ltd, UK).

2.1.3 Plasmids and bacterial strains

The plasmids used in this study are described in Figure 2.1. Plasmids used for transient gene expression studies were amplified in *E. coli* GM2163 (New England Biolabs). *E. coli* XL-1 Blue (Stratagene) was used for the amplification of all other plasmids.

2.1.4 Liquid and solid bacterial growth media

TYN medium (1% (w/v) bacto-tryptone, 1% (w/v) bacto-yeast extract (both supplied by Difco Laboratories, Michigan, USA), 0.5% (w/v) NaCl), supplemented with the appropriate antibiotic, was used for the culture of *E. coli*. For the plating out of *E. coli*, TYN-agar was prepared by adding 1.5 g l^{-1} agar (Difco) to TYN medium prior to sterilisation. Antibiotics were added after sterilisation when the solution had cooled to 50°C.

2.1.5 Antibiotics

Antibiotics were supplied by Sigma Chemical (UK). Ampicillin was dissolved in distilled water (25 mg ml^{-1}), filter-sterilised and used at a final concentration of 50 µg ml^{-1}. Chloramphenicol, used for the growth of *E. coli* GM2163, was dissolved in EtOH (20 mg ml^{-1}) and used at a final concentration of 10 µg ml^{-1}.

2.1.6 DNA modifying enzymes

All restriction enzymes were purchased from Life Technologies, Paisley, Strathclyde together with their reaction buffers, which were provided at a 10 x concentration.

2.2 General Laboratory Procedures

2.2.1 pH measurement

The pH of solutions other than phenol was measured using a Corning pH meter 220 and combination electrode (Corning Incorporation, New York, USA). The pH of phenol-containing solutions were measured using Whatman Narrow Range pH paper (pH 6.0-8.0) (Whatman, Maidstone, UK).

2.2.2 Autoclaving

Equipment and solutions were sterilised at 15 psi for 20 min. Small batches were sterilised in a Prestige High Dome pressure cooker, with larger batches being done in a Laboratory Thermal Equipment Autoclave 225E.

2.2.3 Filter sterilisation

Heat-labile solutions were sterilised by passing them through a Nalgene filter (pore diameter 0.2 μm) into a sterile receptacle.

2.2.4 Glassware

Glassware was sterilised by baking in an oven at 180°C for at least 12 h.

2.2.5 Solutions and equipment for RNA work

Solutions for RNA work were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC, Sigma) overnight and then autoclaved. Sterilised glassware and plasticware were used throughout.

2.3 Plant Material

2.3.1 Growth and harvesting of plants

Seeds of *Arabidopsis thaliana* cv Landsberg *erecta* were obtained from the *Arabidopsis* Stock Centre (Nottingham, UK). *Arabidopsis* seeds were stored at room temperature and germinated in pots containing water soaked ICI potting compost. Pots were covered with clingfilm and placed in the dark for 3 to 4 days at 4°C in order to break seed dormancy. Plants were then grown in a low fluence rate of white light (20 μmol m^{-2} s^{-1}) at 20°C.

Pisum sativum cv Feltham First seeds (pea) and *Phaseolus vulgaris* cv Tendergreen seeds (french bean) were supplied by Sharps International, Sleaford. Seeds were stored at 4°C and germinated on water soaked compost. Plants were grown in continuous white light (100 μmol m^{-2} s^{-1}) at a temperature of 20°C unless otherwise stated. For the isolation of RNA, tissue was harvested directly into liquid

nitrogen and stored at –80°C until use. For membrane isolation, tissue was placed at 4°C and used immediately.

2.3.2 Growth and harvesting of *Arabidopsis* cells

The *Arabidopsis* cell and callus cultures were supplied by Dr Mike May (Department of Plant Sciences, University of Oxford). Cells were grown photomixotrophically in 500 ml flasks containing 200 ml culture medium (1 x Murashige-Skoog salts with minimal organics, 0.5 mg l^{-1} α-naphthaleneacetic acid, 0.05 mg l^{-1} kinetin (all obtained from Sigma), 3% (w/v) sucrose, pH 5.8). Suspension cultures were grown at 20°C in a continuous low fluence rate of white light (20 μmol m^{-2} s^{-1}) with constant shaking (110 rpm) and subcultured every week by a 1/10 dilution. Callus cultures were grown on culture medium supplemented with 0.8% (w/v) agar (Sigma) and transferred to fresh growth medium every 2 weeks.

For large scale isolation of RNA, cells were collected onto 3 MM filter paper (Whatman) by vacuum filtration and frozen in liquid nitrogen. For small scale RNA preparations, 2 ml of the *Arabidopsis* cell culture was transferred to a 2 ml Eppendorf® tube and microcentrifuged for 10 min. The supernatant was removed by aspiration and the pellet immediately frozen in liquid nitrogen. Samples were then stored at –80°C until use.

2.3.3 Monitoring cell culture contamination

Bacterial and fungal contamination of the *Arabidopsis* cell culture was monitored periodically (every 3 months). Cell aliquots (2 ml) were aseptically transferred to 9 cm Petri dishes containing 1.5% (w/v) nutrient agar (Sigma) or czapek-dox agar (1 x czapek-dox broth (Sigma), 1.5% (w/v) agar) and the plates sealed with Micropore® tape (3M Health Care, Loughborough, UK). The plates were placed at 37°C overnight and then examined for contamination. In the event of bacterial/fungal growth cell

cultures were discarded. If necessary, new cultures were established from friable callus.

2.3.4 Treatment of *Arabidopsis* cells

On the third day after subculturing (the calculated point at which cells entered exponential growth phase), cell aliquots (10 ml) were aseptically transferred to 50 ml tissue culture flasks (Nunclon™) which were found to transmit both visible and UV light. Cells were then treated, with constant shaking (80 rpm), for the times indicated in the results.

Chemicals agonists/antagonists, except okadaic acid, cantharidin, A23187, Bay K8644, dibutyryl-cGMP, staurosporine, K252a, genistein, cholera toxin and 5-nitro-2,3-phenylpropylaminobenzoic acid (NPPB), were obtained from Sigma. NPPB was purchased from LC Laborotories (UK). Okadaic acid, cantharidin, staurosporine, K252a, genistein, A23187, Bay K8644, and dibutyryl-cGMP were obtained from Calbiochem-Novabiochem Ltd (UK). Cholera toxin was obtained from GIBCO BRL.

Okadaic acid, staurosporine and K252a were dissolved in dimethyl sulphoxide (DMSO) to yield 1 mM stock solutions. Nifedipine, W-5, W-7, trifluoperazine, A23187, Bay K8644 and NPPB were dissolved in DMSO at 10 mM. Genistein and cantharidin were dissolved in DMSO to yield a stock concentration of 100 mM. Ruthenium red, verapamil, EGTA, lanthanum chloride, tetraethylammonium chloride (TEA), sodium nitroprusside (SNP), phenylacetic acid (PAA), salicylhydroxamic acid (SHAM), NaN_3 and cycloheximide were dissolved in dH_2O at 10 mM (ruthenium red, verapamil, SNP, NaN_3 and cycloheximide), 100 mM (EGTA, PAA and SHAM) or 1M (lanthanum chloride and TEA). Sodium orthovanadate was prepared as described by Schwartz *et al.* (1991) to yield a stock solution of 10 mM. Cholera toxin was dissolved in dH_2O to give a stock solution of 1mg ml^{-1} and thiol-activated before experiments according to the company instructions. Jasmonic acid was dissolved in ethanol to yield a 100 mM stock.

Chemicals were added to 10 ml cell aliquots at the required concentration indicated in the results. Controls were treated with an equivalent amount of the appropriate solvent. In experiments involving dibutyryl-cGMP, prior to addition, the pH of the culture medium was lowered to pH 4.0-4.5 with 50 mM HCl, to facilitate uptake into cells (Bowler *et al.* 1994a).

2.3.5 Establishing the *hy4* cell suspension culture

2.3.5.1 Surface-sterilisation of seed

Arabidopsis hy4-2.23N mutant seeds (obtained from the *Arabidopsis* Stock Centre, Nottingham, UK) were placed onto Whatman filter paper (9 cm diameter) and the paper folded into quarters and once more to form a packet. The packet was then secured with a plastic-coated paper clip, immersed in 70% (v/v) EtOH in a Magenta jar for 2 min and allowed to drain before being transferred to a Magenta jar containing 10% sodium hypochlorite (1.4% (w/v) available chlorine), 0.02% (v/v) Triton X-100 for 15 min. Further manipulations were then carried out in a sterile flow hood. The packet was transferred to a Magenta jar containing sterile dH_2O for 5 min. This was repeated 5 times and the packet dried on a Magenta jar lid for at least 3 h. Seeds were then stored in a petri dish sealed with Micropore® tape.

2.3.5.2 Growth of *hy4* plants

In a sterile flow hood, surface-sterilised seeds were sown onto Petri dishes containing germination medium (1 x Murashige and Skoog salts (Sigma), 2% (w/v) sucrose, 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, 0.5 g l^{-1} MES pH 5.7). Seeds were placed in the dark at 4°C for 3 days in order to break seed dormancy and then grown in a low fluence rate of white light (20 µmol m^{-2} s^{-1}) for 21 days.

2.3.5.3 Callus induction

In a sterile flow hood, three week old *hy4* plants were transferred to a glass plate which had been previously rinsed in ethanol and left to dry. Leaf tissue was cut into segments with a sterile scalpel blade and transferred to petri dishes containing callus induction medium (2% (w/v) glucose, 0.8% (w/v) agar, 1 x Gamborg B5 salts, 0.5 mg l^{-1} 2,4-D, 0.05 mg l^{-1} kinetin, 0.5 g l^{-1} MES pH 5.7). Petri dishes were then sealed with Micropore® tape and placed in continuous white light (20 µmol m^{-2} s^{-1}) for 2-3 weeks until friable callus was obtained.

2.3.5.4 Initiation of *hy4* suspension cultures

After callus formation, pieces of friable callus (0.3 g) were transferred to a 100 ml conical flask containing 20 ml sterile culture medium (1 x Murashige and Skoog salts, 0.5 mg l^{-1} α-naphatheleneacetic acid, 0.05 mg l^{-1} kinetin, 3% (w/v) sucrose, pH 5.8). The flask was then shaken at 110 rpm in continuous white light (20 µmol m^{-2} s^{-1}) at 18-20°C. After 7-10 days, the medium was decanted, replaced with 50 ml of fresh culture medium and the suspension transferred to a sterile 250 ml conical flask. When the volume of tissue had doubled, the medium was decanted and replaced with 100 ml fresh culture medium. The *hy4* cell culture was then subcultured, once a week, by transferring 30% of the culture to a fresh flask containing 70 ml culture medium. For gene expression studies, *hy4* cells were used on the seventh day after subculture.

2.4 Illumination of Plant Material

2.4.1 Light sources

Illuminations were carried out in controlled environment rooms at 21°C. The spectra of all the light qualities were measured with a spectroradiometer (Macam SR9910) and are shown in Figure 2.2. White light was provided by warm white fluorescent tubes

(Osram, Münich, Germany), UV-A/blue light was provided by Sylvania 40 W T12 blue fluorescent tubes (GTE Sylvania, Shipley, UK) and red light was obtained by covering the white fluorescent tubes with orange Cinemoid. UV-A light was provided by Sylvania F35W/B1-2B blacklight-blue fluorescent tubes emitting wavelengths between 320 and 390 nm. UV-B light was obtained by covering Phillips TL 40W/12 ultra-violet fluorescent tubes with cellulose acetate (to omit UV-C), which was changed every 24 h. Far-red light was provided by Toshiba FL20S FR-74 tubes. The required fluence rate was achieved by varying the number of tubes in the growth area and adjusting the distance of the plant material from the light source. The fluence rates of the light regimes used are described for each experiment. In the appropriate experiments, wavelengths below 320 nm were removed by covering tubes with a clear polyester 130 filter (Lee Filters, Andover, UK). Wavelengths below 390 nm were removed using a clear polyester 226 filter (Lee Filters).

2.4.2 Fluence rate measurement

The photon fluence rates of the visible light sources were measured using a Li-Cor quantum sensor, model Li-185B. The photon fluence rates of the UV light sources were measured with a spectroradiometer (Macam SR9910)

2.5 Isolation of Total RNA from Plant Material

2.5.1 Preparation of phenol

Five hundred grams of phenol (detached crystals) was melted at 65°C for 30 min and 0.58 g of 8-hydroxyquinoline added before the phenol had cooled. The warm solution was saturated with 100 mM Tris-HCl (pH 8.0) and mixed vigorously for 10 min. After allowing the solution to settle, the upper phase was removed by aspiration and the pH of the phenol measured. Aspiration and washing with 100 mM Tris-HCl (pH 8.0)

was repeated until the pH of the phenol was greater than 7.6. The upper phase was then replaced with 100 ml of 10 mM Tris-HCl (pH 7.6) and the solution stored at 4°C.

2.5.2 Large scale preparation of RNA

RNA was extracted using the method of Jackson *et al.* (1995). Approximately 0.5 g of frozen plant or cell tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was then transferred to a 15 ml Corex® tube containing 4 ml extraction buffer (5 M guanidine thiocyanate (Sigma), 25 mM tri-sodium citrate, 2 mM EDTA (disodium salt), 50 mM Tris-HCl pH 7.6, 0.5% (w/v) sarcosyl (Sigma), 1 mM β-mercaptoethanol) and briefly vortexed. Equal volumes (4 ml) of phenol and chloroform were added and the solution vortexed once more before spinning at 3,000 *g* for 10 min at 4°C. After centrifugation, the upper layer was removed to a clean tube, 2 volumes of EtOH added and the tube inverted several times before being transferred to –20°C for 2-12 h.

After precipitation, RNA was pelleted by centrifugation at 3,000 *g* for 10 min at 4°C and the supernatant discarded. The pellet was washed with 1 ml 3 M NaOAc (pH 5.5) and vortexed briefly to resuspend the pellet. After centrifugation for 5 min, the pellet was washed in 1 ml 70% (v/v) EtOH then spun once more for 10 min. The resulting pellet was dried under a vacuum and dissolved in an appropriate volume of DEPC-treated dH_2O.

The purity of the preparation was estimated by measuring the OD at 260 nm and 280 nm. A pure preparation was taken as having a 260/280 nm ratio of 1.8-2.0. The integrity of the RNA was examined on a 1.5% (w/v) agarose mini-gel. All RNA preparations were stored at –80°C.

2.5.3 Small scale isolation of RNA from *Arabidopsis* cells

Cell tissue, harvested as described in 2.3.2, was ground to a fine powder in liquid nitrogen using a small mortar and pestle, transferred to a sterile 2 ml Eppendorf® tube containing 0.4 ml extraction buffer (as above) and vortexed for 10 s. Equal volumes of phenol and chloroform (0.4 ml) were added and the solution vortexed for 20 s before spinning at 10,000 *g* for 10 min at 4°C. The upper layer was removed to a clean tube, 2 volumes of EtOH added and the solution mixed before being transferred to -20°C for 2-12 h.

After precipitation, the tube was microcentrifuged for 10 min at 4°C and the supernatant discarded. The pellet was washed with 0.2 ml 3 M NaOAc (pH 5.5) and then 0.2 ml 70% (v/v) EtOH. The resulting pellet was dried under a vacuum and dissolved in DEPC-treated dH_2O.

2.5.4 Isolation of RNA from *Arabidopsis* protoplasts

A 6 ml protoplast suspension (approximately 2 x 10^5 protoplasts) was harvested as described in 2.15.1. Grinding of the protoplasts was not neccessary and total RNA was extracted as described in 2.5.3.

2.6 Amplification and Preparation of Plasmid DNA

2.6.1 Preparation of competent cells

Competent cells were prepared by the method described by Sambrook *et al.*, (1989). *E. coli* was grown overnight in 10 ml TYN medium at 37°C with constant shaking. One hundred ml of fresh TYN medium was inoculated with 5 ml of the overnight culture and shaken at 37°C until an OD at 550 nm of 0.35 was reached. Seventy ml of the suspension was transferred to two sterile 35 ml centrifuge tubes and chilled on ice for 15 min. Cells were then pelleted at 2,000 *g* for 5 min at 4°C and the supernatant

decanted. The bacterial pellet was resuspended in a total volume of 10.5 ml ice-cold TFB 1 (100 mM RbCl, 50 mM $MnCl_2$, 30 mM KOAc, 10 mM $CaCl_2$, 15% (v/v) glycerol) and kept on ice for 90 min. After incubation, the cell suspension was centrifuged as before and resuspended in 2.8 ml ice-cold TFB 2 (10 mM MOPS (Sigma), 10 mM RbCl, 75 mM $CaCl_2$, 15% (v/v) glycerol, pH 7.0). Cells were then separated into 0.2 ml alquiots, in 1.5 ml Eppendorf® tubes, frozen in liquid nitrogen and stored at –80 °C until use.

2.6.2 Transformation of competent cells

Frozen competent cells prepared as described in 2.6.1 were thawed at room temperature and placed on ice. Approximately 200 ng of plasmid DNA, in a volume of 5-10 µl, was added to the Eppendorf® and the tube gently shaken before being returned to ice for 20 min. After this period, the cells were heat-shocked at 37°C for 1 min and returned to ice for a further 2 min. TYN medium (0.8 ml) was added to the cell suspension and the Eppendorf® shaken at 37°C for 50 min to allow expression of the antiboitic resistance encoded on the plasmid. Cells were then pelleted by centrifugation at 10,000 *g* for 30 s, the supernatant discarded, and the pellet resuspended in 0.1 ml TYN medium. One hundred µl serial 1/10 dilutions of the cell suspension were plated on agar with the appropriate antibiotic. The plates were allowed to dry and placed at 37°C overnight. As a control, transformed cells were plated on agar containing no antibiotic. A second control was also performed where competent cells were treated, as above, in the absence of plasmid DNA and plated on antibiotic-containing plates.

2.6.3 Small scale preparation of plasmid DNA

For this method the Wizard™ Minipreps DNA Purification System (Promega) was used in accordance with the manufacturer's instructions. A single bacterial colony was used to inoculate 3.0 ml of TYN medium, supplemented with the appropriate antibiotic, in a

7 ml bijou tube. The culture was grown overnight with constant shaking at 37°C. One ml of the overnight culture was transferred to a 1.5 ml Eppendorf® tube and centrifuged at 10,000 *g* for 5 min. The supernatant was discarded and the bacterial pellet resuspended by vortexing in 200 ml of cell resuspension solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 mg ml^{-1} RNAse A). Once fully resuspended, 200 ml of cell lysis solution (0.2 M NaOH, 1% (w/v) SDS) was added and the solution mixed by gentle inversion. Next, 200 ml neutralisation solution (1.32 M KOAc pH 4.8) was added and the solution mixed as before. The tube was centrifuged at 10,000 *g* for 5 min and the supernatant removed to a 2.0 ml Eppendorf® tube. One ml of the Wizard™ Minipreps DNA purification resin was added and the solution gently mixed. Meanwhile, a 3 ml disposable syringe was attatched to a minicolumn and placed onto a vacuum manifold (Promega). The resin/DNA mixture was then transferred to the syringe and a vacuum applied to pull the slurry into the minicolumn. The vacuum was broken, 2 ml of wash solution (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 2.5 mM EDTA, 55% (v/v) EtOH) added and the vacuum reapplied. Once the wash solution had passed through, the column was left to dry for 2 min. The vacuum was broken and the minicolumn removed from the syringe and transferred to a 1.5 ml Eppendorf® tube. This was microcentrifuged for 20 s to further dry the resin. The minicolumn was then transferred to a clean 1.5 ml Eppendorf®, 50 µl of TE (pH 7.6) added and then left at room temperature for 1 min. Plasmid DNA was eluted by microcentrifugation for 20 s. The minicolumn was then discarded.

2.6.4 Large scale preparation of plasmid DNA

Two methods were employed to prepare plasmid DNA for transient gene expression studies. These are described in 2.6.4.2 and 2.6.4.3. After purification, plasmid DNA was analysed by restriction digestion (2.11) with the appropriate restriction enzymes prior to protoplast transfection.

2.6.4.1 Growth of bacterial cultures

A single bacterial colony containing the plasmid of choice was used to inoculate 10 ml of TYN medium, containing the appropriate antibiotic, and grown overnight at 37°C with constant shaking. TYN medium (500 ml), supplemented with the appropriate antibiotic, was inoculated with the mini-culture and shaken at 37°C for 24 h. The culture was then decanted into sterile 250 ml centrifuge bottles and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was discarded and the cell pellet used for plasmid DNA preparation.

2.6.4.2 Preparation of plasmid DNA by equilibrium centrifugation in a CsCl-EtBr gradient

The cell pellet, prepared as in 2.6.4.1, was resuspended in 20 ml of Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA). Two hundred mg of lysozyme (48,000 units mg^{-1}, Sigma) was dissolved in 2 ml of Solution I, added to the cell suspension and the solution incubated at room temperature for 10 min. Next, 40 ml of Solution II (0.2 M NaOH, 1% (w/v) SDS) was added, the suspension gently mixed and kept on ice for 10 min. After incubation, 20 ml of 3 M KOAc (pH 4.8) was added and the suspension mixed by inversion prior to incubation on ice for a further 30 min. The mixture was then centrifuged at 2,000 *g* for 10 min at 4°C and the supernatant transferred to another 250 ml centrifuge bottle through 1 layer of muslin. Fifty ml of *iso*-propanol was added to the filtrate, the phases mixed and left at room temperature for 5 min. DNA and RNA were pelleted by centrifugation at 5,000 *g* for 10 min at 4°C and the supernatant discarded. The centrifuge bottle was then inverted to allow the pellet to dry. After drying, the pellet was dissolved in 7.1 ml of TE (pH 8.0) and transferred to an ultracentrifuge tube containing 6.72 g of CsCl. The solution was inverted to mix, 0.425 ml of EtBr (10 mg ml^{-1}) added and left at room temperature for 30 min to allow the CsCl to dissolve. The tube was then centrifuged at 18,000 *g* for 48 h at room temperature. After centrifugation the plasmid band was removed with a

sterile pasteur pipette into a 15ml Corex® tube. An equal volume of CsCl-saturated *iso*-amyl alcohol was added and the phases mixed. The phases were allowed to separate and the upper phase removed. This *iso*-amyl alcohol extraction was repeated until the aqueous phase contained no pink colour. The aqueous phase was then dialysed against a 1000 x volume of TE (pH 8.0) in dialysis tubing (Sigma) for 2 h at room temperature and then overnight against fresh TE (pH 8.0) at 4°C. The dialysis tubing had been boiled for 5 min, in the presence of 0.1% (w/v) SDS, and allowed to cool prior to use. The concentration of the DNA solution was determined as described in 2.7 and diluted to the required concentration then stored in aliquots at -20°C.

2.6.4.3 Preparation of plasmid DNA using the Qiagen mega-plasmid purification system

The Qiagen mega-plasmid purification system (Qiagen) was used in accordance with the manufacturer's instructions. Cells containing the plasmid of choice were prepared as described in 2.6.4.1. The cell pellet was resuspended in 50 ml P1 buffer (100 mg ml^{-1} RNAse A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) and transferred to a clean centrifuge tube. Fifty ml of P2 buffer (200 mM NaOH, 1% (w/v) SDS) was added to the cell suspension and the solution mixed gently by inverting the bottle several times before incubating at room temperature for 5 min. Next, 50 ml of P3 buffer (3 M KOAc pH 5.5) was added and the solution incubated on ice for 30 min after mixing by inversion. The suspension was then centrifuged at 4°C for 5 min at 20,000 *g*. Meanwhile, a Qiagen column 2,500 was equilibrated by applying 35 ml QBT buffer (750 mM NaCl, 50 mM MOPS, 15% (v/v) EtOH, 0.15% (v/v) Triton X-100, pH 7.0) and the column allowed to empty by gravity flow. The supernatant obtained after centrifugation was then applied to the column and allowed to enter the resin by gravity flow. The column was then washed with 4 x 50 ml QC buffer (1 M NaCl, 50 mM Tris-HCl, 15% (v/v) EtOH, pH 8.5). Plasmid DNA was eluted into a sterile 200 ml centrifuge bottle by the addition of 35 ml QF buffer (1.25 NaCl, 50 mM Tris-HCl,

15% (v/v) EtOH, pH 8.5). The DNA was precipitated by adding 0.7 volumes of *iso*-propanol, the solution mixed, transferred to 2 x 35 ml centrifuge tubes and spun at 20,000 *g* for 30 min at 4°C as above. The supernatant was discarded and the DNA pellets washed in 5 ml of ice-cold 70% (v/v) EtOH and centrifuged once more. After centrifugation, the supernatant was removed and the pellets air-dried before redissolving in a suitable volume of TE (pH 7.6). The concentration of the DNA solution was determined as described in 2.7 and diluted to the required concentration before storing at –20°C.

2.7 Quantification of DNA and RNA

An aliquot of the nucleic acid solution to be quantified (usually 5-10 µl) was diluted to 1 ml with DEPC-treated dH_2O. The absorbance of the solution was measured between 220 nm and 320 nm against a DEPC-treated dH_2O blank. An absorbance at 260 nm of 1 was taken to indicate the following concentrations:

Form of nucleic acid	Concentration (µg ml^{-1})
Double stranded DNA	50
Single stranded DNA and RNA	40

2.8 Agarose Gel Electrophoresis

All agarose (*ultra* PURE™) was supplied by Life Technologies, Paisley.

2.8.1 Electrophoresis of DNA

The appropriate concentration of agarose (0.5-2.0% (w/v)) was added to the volume of 1 x TBE (0.09 M Tris-borate, 2 mM EDTA) required for the gel being cast. The agarose suspension was heated in a microwave until the agarose had completely dissolved. The solution was allowed to cool to around 60°C at which point 10 mg ml^{-1}

EtBr was added to a final concentration of 1 mg ml^{-1} and the gel solution poured into the electrophoresis apparatus. After allowing the gel to set for 30 min, enough 1 x TBE running buffer was added to just submerge the gel. DNA samples to be loaded were mixed with 1/10 volume of loading buffer (20% (v/v) glycerol, 0.025 M NaOH, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF) and loaded with a pipette. Electrophoresis was carried out at 15-80 mA until the bromophenol blue had migrated two-thirds of the way down the gel. The gel was then visualised under UV light (Spectroline® transilluminator, Model TC-312A).

2.8.2 Non-denaturing electrophoresis of RNA

Non-denaturing electrophoresis of RNA was carried out as described in 2.8.1 for DNA.

2.8.3 Denaturing electrophoresis of RNA

This method was used for gels that were to be blotted for hybridisation analysis. The appropriate amount of agarose (1.3-1.5 g) was added to 80 ml of DEPC-treated dH_2O. The agarose suspension was then heated in a microwave until the agarose had dissolved. Once the agarose solution had cooled to 60°C, 10 ml of formaldehyde (37% (v/v), Sigma) and 10 ml 10 x MOPS buffer (0.2 M MOPS (Sigma), 0.05 M NaOAc, 0.01 M EDTA, pH 7.0) was added and the gel mixed by swirling prior to pouring into the electrophoresis apparatus. After a period of 30 min the gel had set and was transferred to 4°C for another 30 min. The gel was then placed into the electrophoresis tank and submerged in 1 x MOPS. RNA (5-20 µg) was prepared in a solution of 70% (v/v) formamide (Fluka Biochemicals, Gillingham, UK), 10 µg ml^{-1} EtBr, 2.3 x MOPS, 2.3% formaldehyde in a volume no greater than 50 µl. This solution was heated to 65°C for 5 min and placed on ice. One-tenth volume of loading buffer (50% (v/v) glycerol, 1 mM EDTA pH 8.0, 0.25% (w/v) bromophenol blue, 0.25% (w/v)

xylene cyanol FF) was added and the RNA samples loaded onto the gel with a pipette. Electrophoresis was carried out at 15-80 mA until the bromophenol blue had migrated two thirds of the distance down the gel. The gel was then visualised under UV light.

2.9 Isolation of DNA Fragments from Agarose Gels

2.9.1 Phenol/chloroform extraction of DNA

The DNA fragment of interest was initially separated from residual DNA by agarose gel electrophoresis as described in 2.8.1 and identified by EtBr staining when viewed under UV light. The fragment was then excised with a clean scalpel blade, cut into small pieces and separated (0.1 g) into 1.5 ml Eppendorf® tubes. Five hundred µl of phenol was added to each Eppendorf® and the mixture vortexed until the gel fragments had emulsified. Each tube was then frozen in liquid nitrogen then immediately centrifuged at 10,000 *g* for 10 min. The upper phases were removed to a fresh Eppendorf® tube and an equal volume of phenol and chloroform added. After vortexing, the solution was centrifuged, as before, for 2 min and the upper layer removed to a clean tube. An equal volume of chloroform was added and the centrifugation step repeated. The upper layer was removed to a fresh tube and 1/10 volume 3 M NaOAc (pH 5.5) added along with 2 volumes of EtOH. DNA was precipitated at –20°C for at least 1 hour then centrifuged at 10,000 *g* for 5 min. After decanting the supernatant, the DNA pellet was washed with 70% (v/v) EtOH before centrifuging once more. The supernatant was removed, the pellet air-dried for 10 min and then resuspended in an appropriate volume of TE (pH 7.6). This procedure yielded about 50-60% recovery of the DNA.

2.9.2 Qiaex gel extraction of DNA

For this method the Qiaex Gel Extraction System (Qiagen) was used in accordance with the manufacturer's instructions. The DNA of interest was isolated from the agarose gel as described in 2.9.1. The gel slice (0.1 g) was transferred to a 1.5 ml Eppendorf® tube, containing 300 ml buffer QX1 (3 M NaI, 4 M $NaClO_4$, 10 mM Tris-HCl pH 7.0, 10 mM sodium thiosulphate). One-tenth volume of 1 M mannitol was added to enable solubilistion of TBE agarose gels. The sample was then incubated at 50°C until the agarose had completely dissolved. Ten ml of Qiaex resin was added and the sample incubated at 50°C for a further 10 min to allow the DNA to bind to the resin. After incubation, the sample was microcentrifuged for 30 s and the supernatant removed with a pipette. The pellet was resuspended in 0.5 ml buffer QX2 (8 M $NaClO_4$, 10 mM Tris-HCl pH 7.0) and the sample centrifuged once again. This wash step was repeated twice with an equal volume of buffer QX3 (70% (v/v) EtOH, 100 mM NaCl, 10 mM Tris-HCl pH 7.5). The final pellet was air-dried for 10 min and resuspended in 20 µl TE (pH 7.6) and incubated at room temperature for 5 min, vortexing periodically, to elute the DNA from the Qiaex resin. The sample was centrifuged as before and the supernatant transferred to a clean tube. This procedure yielded >70% recovery of the DNA.

2.10 Digestion of DNA with Restriction Endonucleases

DNA to be restricted was prepared in a solution of 1 x the appropriate buffer and 1-20 units of the restriction enzyme(s) added ensuring that its concentration did not exceed 10% (v/v). Reactions were incubated at 37°C for 2-24 h and monitored, where sufficient DNA was available, by running an aliquot of the reaction on an agarose gel against uncut and marker DNA (1 kb ladder, Life Technologies).

2.11 Northern Blotting

RNA was separated on a denaturing agarose gel as described in 2.8.3. A wick of Whatman 3 MM paper was placed onto a support and soaked in 20 x SSC (3 M NaCl, 0.3 mM *tri*-sodium citrate). The wick was also dipped into a reservoir of 20 x SSC. The gel was placed on top of the wick with the well side of the gel face down and care taken to remove air bubbles between the wick and the gel. A piece of nylon membrane (Hybond-N, Amersham) was cut to the same size as the gel, rinsed in sterile dH_2O and placed on top of the gel. Any air bubbles between the nylon and the gel were removed using a plastic pipette. Two pieces of Whatman 3 MM paper cut to the size of the gel were rinsed in sterile dH_2O and placed on top of the nylon membrane. The area of the wick within a few mm of the gel was then covered with cling film to prevent short circuiting and a large quantity of paper towels along with a 500 g weight placed on top. The blot was left overnight then disassembled. The gel and filter were examined under UV light to assess the transfer of RNA from the gel to the nylon. After successful transfer, the filter was briefly rinsed in sterile dH_2O and air-dried at room temperature. The dried filter was then wrapped between two pieces of Whatman 3 MM paper and baked at 80°C in order to fix the RNA.

2.12 Radiolabelling of DNA

2.12.1 Preparation of DNA to be labelled

The DNA sequences to be labelled were cut from their host plasmid using the appropriate restriction enzyme(s) and purified as described in 2.9. The amount of the DNA was estimated by running an aliquot of the fragment on an agarose gel as described in 2.8.1 and comparing it to known amounts of DNA loaded onto the same gel.

2.12.2 Random priming of dsDNA

DNA was labelled with [α-^{32}P]dCTP using the Rediprime DNA labelling kit supplied by Amersham International and was used in accordance with the manufacturer's instructions. Approximately 25-50 ng of DNA, prepared as described in 2.12.1, was diluted to a volume of 45 µl with sterile dH_2O in a 1.5 ml Eppendorf® tube. The DNA was denatured by heating the sample to 95°C in a boiling water bath for 5 min and then briefly microcentrifuged. The denatured DNA was added to the labelling mix (dATP, dGTP, dTTP, exonuclease-free 'Klenow' enzyme and random nonamer primers) and the mixture reconstituted by gently flicking the tube until the blue colour of the labelling mix was evenly distributed. After a brief microcentrifugation, 50 µCi (3,000 Ci mmol^{-1}) of [α-^{32}P]dCTP was added to the reaction mixture and the tube incubated at 37°C for 15 min. After this, the reaction was stopped by the addition of 5 µl 0.5 M EDTA and 45 µl sterile dH_2O. For use in hybridisation analysis, DNA was denatured by heating to 95-100°C for 5 min. The sample was chilled on ice until required. This was carried out after assessing the level of incorporation and removal of un-incorporated radionucleotides.

2.12.3 Separation of labelled DNA from un-incorporated radionucleotides using spin columns

A sterile 1 ml syringe barrel was plugged with siliconised glass wool and filled with Sephadex TESN previously made by autoclaving Sephadex G50 medium (Pharmacia, Milton-Keynes, UK) in a 20-fold volume of TESN (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 M NaCl, 0.05% (w/v) SDS). The column was spun in a 15 ml Corex® tube at 700 *g* for 4 min at room temperature and the process repeated until the column volume was 1 ml. One hundred µl of TESN was loaded on top of the column and the column then centrifuged as before. The TESN wash was repeated once more prior to transferring the solution of labelled DNA and un-incorporated nucleotides onto the column. A clean screw-top Eppendorf® tube was placed under the column to collect

the eluate and the column centrifuged once more. The DNA obtained was stored at 4°C until use and the column disposed of.

2.12.4 Measurement of the incorporation of radionucleotides into DNA probes

The level of incorporation of radioactivity into DNA probes was estimated by removing 2 µl aliquots of the probe solution before and after centrifugation through the Sephadex column described above. The 2 µl samples were placed into screw-cap Eppendorf® tubes containing 0.5 ml dH_2O. These were then inserted into separate scintillation vials and the radioactivity counted (LKB 1209 rackbeta scintillation counter). The percentage of incorporation could then be determined by comparing the counts of the two radiolabelled sample aliquots before and after the centrifugation step: (counts after centrifugation/counts before centrifugation) x 100. The specific activity of the labelled probe (cpm incorporated per µg DNA) could then be calculated, given that the amount of radiolabelled deoxy-nucleotide, template DNA and percentage incorporation of the radiolabel were known. A typical Rediprime reaction with an incorporation of 60% yielded a specific activity of 1.7×10^9 cpm μg^{-1}.

2.13 Hybridisation Analysis of Northern Blots

2.13.1 Hybridisation analysis using homologous probes

Northern blots were analysed using the method of Church and Gilbert (1984). Nylon filters, prepared as in 2.11, were pre-hybridised at 55°C in a shaking water bath or hybridisation oven for 1-2 h. Enough pre-hybridisation solution (0.5 M Na_2HPO_4 pH 7.2, 7% (w/v) SDS, 10 mg/ml BSA) was used to cover the membrane filter. The radiolabelled cDNA probe was denatured by heating at 95°C for 3 min and chilled on ice. The denatured probe was then added to the pre-hybridisation solution using an

automatic pipette. Care was taken to remove any air bubbles from the sealed bag or container. Hybridisation was carried out at 55°C for 16 h.

2.13.2 Hybridisation analysis using heterologous probes

Northern blots were analysed using the method of Sambrook *et al.* (1989). Nylon filters, prepared as in 2.11, were pre-hybridised at 37°C in a shaking water bath or hybridisation oven for 4 h. Enough prehybridisation solution (5 x (v/v) Denhardts solution (0.1% (w/v) Ficoll 400, 0.1% (w/v) PVP 360, 0.1% BSA , all from Sigma), 5 x SSC, 50% formamide, 100-200 μg ml^{-1} denatured, sonicated salmon sperm DNA (Sigma), 0.1% (w/v) SDS) was used to cover the filter(s) in a sealed bag, plastic box or hybridisation oven container. The radiolabelled cDNA probe was denatured as above and then added to the pre-hybridisation solution using an automatic pipette. Hybridisation was carried out at 37°C for 16 h.

2.13.3 Washing of northern blots

After hybridisation with homologous probes, filters were washed initially in an appropriate volume of 2 x SSC, 1% (w/v) SDS at 55°C in a shaking water bath. The filter was then washed at increasing stringency depending on the amount of radioactivity bound to the membrane. Each wash was for 10 min unless otherwise stated.

After hybridisation with heterologous probes, filters were washed initially in an appropriate volume of 2 x SSC, 0.1% (w/v) SDS at 37°C in a shaking water bath. The filter was then washed at increasing stringency as described above. All filters were rinsed in 2 x SSC before autoradiographing.

2.13.4 Autoradiography

Filters to be autoradiographed were wrapped in cling film and exposed to Fuji X-ray film (type RX) in a film cassette with intensifying at –80°C for the appropriate length of time.

2.13.5 Stripping filters of bound radiolabelled probes and blocking agents

After hybridisation and autoradiography, bound probe and blocking agents were removed by pouring a boiling solution of 0.1% (w/v) SDS over the filter. The solution was allowed to cool to room temperature, and the process repeated a further two times. After this, the filter was wrapped in cling film and autoradiographed overnight to verify that the radiolabelled probe had been removed. The presence of RNA on the filter after stripping could then be detected as described below.

2.13.6 Staining of filter-bound RNA with Methylene Blue

This procedure was carried out as described by Sambrook *et al.* (1989). The filter containing the bound RNA was placed in 0.04% (w/v) Methylene Blue, 0.5 M NaOAc (pH 5.2) for 5 to 10 min. The filter was then washed in DEPC-treated dH_2O until the RNA could be visualised as blue bands on a white background. The stain was removed by washing the filter in 20% (v/v) acetic acid until the bands were no longer visible.

2.14 Antibody Studies

2.14.1 Membrane protein isolation

Membrane proteins were isolated by the method of Gallagher *et al.* (1988). Plant material was grown and harvested as described in 2.3.1. All manipulations were carried out at 4°C. Manipulations involving dark-grown tissue were carried out under a dim green safe light. Harvested tissue (1-5 g) was placed into a chilled mortar on ice and ground with a pestle in 5 ml of chilled homogenisation buffer (30 mM Tris-Mes pH 8.0, 0.4 M sucrose, 10 mM KCl, 1 mM EDTA, 0.1 mM $MgCl_2$, 1 mM dithiothreitol). The homogenate was filtered through a 70 µm nylon mesh cloth and centrifuged at 6,000 *g* for 10 min at 4°C. The supernatant was removed to an ultracentrifuge tube and spun at 100,000 *g* for 30 min at 4°C to yield a soluble fraction and membrane pellet. The pellet was resuspended in 100-200 µl resuspension buffer (10 mM Tris-Mes pH 7.2, 1 mM dithiothreitol, 0.25 M sucrose) to give a protein concentration of 1-20 mg ml^{-1}. The membrane fraction was either used immediately or stored at –80°C in 20 µl aliquots. Protein concentrations were determined as described in 2.19.

2.14.2 Polyacrylamide gel electrophoresis

Membrane proteins were separated on a 12.5% (w/v) polyacrylamide gel in the presence of SDS by the method of Laemmli (1970). The required volume for the resolving gel (375 mM Tris-HCl pH 8.8, 12.5% (w/v) acrylamide (at an acrylamide:bis-acrylamide ratio of 30:0.8), 0.033% (v/v) N,N,N',N'-tetramethylene diamine, 0.05% (w/v) ammonium persulphate and 0.1% (w/v) SDS) was prepared in a conical flask and poured in a vertical slab format using a 25 ml pipette. The acrylamide solution was then covered with *iso*-propanol and allowed to set for 10-20 min at room temperature. Once the gel had set, the alcohol was removed and the gel rinsed several times with dH_2O to remove any unpolymerised acrylamide. The required volume of stacking gel (125 mM Tris-HCl pH 6.8, 3% (w/v) acrylamide (30:0.08), 0.067% (v/v)

N,N,N',N'-tetramethylene diamine, 0.1% (w/v) ammonium persulphate and 0.1% (w/v) SDS) was prepared and poured onto the resolving gel using a 5 ml pipette. The well comb was inserted and the gel left to set as before. Once set, the gel was mounted into the electrophoresis apparatus and electrophoresis buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS, pH 8.3) added to the top and bottom reservoirs.

The protein samples (80 µg) were prepared by heating them to 100°C for 5 min in 1 x SDS gel-loading buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (w/v) glycerol), in a total volume of 80 µl, and chilled on ice. Protein samples were then loaded (20-30 µl) into the wells of the gel and electrophoresis was carried out at 100 mA, with cooling, until the bromophenol blue had migrated to the bottom of the resolving gel. For western blotting, 16 µg protein was loaded onto the gel. The molecular weight markers used were: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

2.14.3 Staining polyacrylamide gels with Coomassie brilliant blue

After electrophoresis, the gel was immersed in at least 5 volumes of staining solution (0.1% (w/v) Coomassie brilliant blue G250, 50% (v/v) methanol, 10% (v/v) acetic acid) for at least 2 h at room temperature with gentle shaking. The staining solution was then removed and the gel destained in 10% (v/v) methanol, 10% (v/v) acetic acid for at least 4 h at room temperature with gentle shaking. The destain solution was changed 3 or 4 times over this period. Gels were then stored indefinitely in dH_2O in a sealed plastic bag.

2.14.4 Western blotting

Proteins were electrotransferred from SDS-polyacrylamide gels to a nitrocellulose filter (Hybond-C, Amersham) using a electroblotting apparatus (Bio-Rad, Hertfordshire, UK). The graphite plates were rinsed in dH_2O and dried using nonabsorbant tissues. Two pieces of Whatman 3 MM paper and one piece of nitrocellulose were cut to the same size of the resolving gel and soaked in electrophoresis transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% (w/v) SDS, 20% (v/v) methanol). One piece of filter paper was placed onto a graphite plate and care taken to ensure that any air bubbles were removed using a glass pipette. The nitrocellulose filter was then placed on top followed by the separation gel which had been briefly rinsed in dH_2O. The remaining piece of filter paper was placed on top followed by the second graphite plate, the apparatus closed and then inserted into the electrophoresis tank. The tank was filled with transfer buffer and electrophoresis carried out at 350 mA for 4 h. The filter was either stained with amido black or used for immunological probing. The gel was stained as in 2.14.3 to check that transfer was complete.

2.14.5 Staining proteins immobilised on nitrocellulose filters

The nitrocellulose filter prepared as described in 2.14.4 was allowed to dry at room temperature for 30-60 min before staining in 0.1% (v/v) amido black 10B, 5% (v/v) acetic acid for 30-60 s. The filter was then destained in 45% (v/v) methanol, 5% (v/v) acetic acid until proteins were visualised as purple bands on a white background. Filters were then stored in a sealed plastic bag.

2.14.6 Binding of immunological probes

The anti-serum used in this study was kindly provided by Professor Graeme Milligan (Division of Biochemistry and Molecular Biology, University of Glasgow). The nitrocellulose filter prepared as described in 2.14.4 was blocked overnight in 100 ml

BIBT buffer (0.2 M Tris base, 1.5 M NaCl, 0.5% (v/v) Tween 20) with constant shaking. The solution was then decanted and the filter incubated in 30 ml BIBT buffer, containing 5 % (v/v) normal (donkey) sera and a 1/150 dilution of anti-sera, at room temperature for 90 min with constant shaking. The blot was then washed 4 times in 30 ml BIBT buffer before being given a final wash in 30 ml BIB buffer (0.2 M Tris base, 1.5 M NaCl). All washes were for 12 min at room temperature. The filter was then incubated in 30 ml BIB buffer, containing 5% (v/v) normal sera and a 1/200 dilution of horseradish peroxidase (HRP)-conjugate, for 90 min at room temperature with gentle agitation. As before, the filter was washed 5 times in 30 ml BIB buffer. The filter was developed using chloronaphthol as a substrate for HRP. An aliquot (150 µl) of 4% (v/v) H_2O_2 was added to 50 ml Tris-HCl (pH 7.5) followed by 10 ml chloronaphthol (30 mg in 10 ml methanol). The solution was mixed and added directly to the filter. The reaction was allowed to proceed at room temperature, with gentle agitation, until bands of the desired intensity could be visualised. The reaction was stopped by washing the filter briefly in dH_2O. Filters were left to air dry at room temperature and then stored wrapped in aluminium foil.

2.15 Protoplast Isolation

Unless otherwise stated, cut off pipette tips were used in all manipulations involving protoplasts.

2.15.1 Isolation of *Arabidopsis* cell culture protoplasts

This method is a modified version of that obtained from Dr Bernd Weisshaar (Max-Planck-Institut, Köln, Germany). The *Arabidopsis* cell culture was used on the third day after subculture unless otherwise stated. Fifty ml of cells were aspectically transferred to a 50 ml centrifuge tube. Cells were pelleted at 150 *g* for 5 min and the supernatant discarded. The cell pellet was resuspended in 40 ml 0.24 M $CaCl_2$ and centrifuged as before. After decanting the supernatant, cells were resuspended in 60 ml

of digestion medium (1% (w/v) cellulase R10, 0.25% (w/v) macerozyme R10 (both from Yakult Honsha, Japan), 0.24 M $CaCl_2$) and poured into a 13.5 cm diameter Petri dish. The cells were then incubated for 16 h in darkness at room temperature with constant shaking (30 rpm). It was subsequently established that the enzyme concentration in the digestion medium affected transient gene expression. Therefore, the protocol was modified accordingly as described in chapter 5. After digestion of the cell wall, cell debris was removed by filtering the protoplasts through one layer of muslin and a 100 µm steel seive (Sigma) into 2 x 50 ml centrifuge tubes. Protoplasts were centrifuged as above, washed in 25 ml of 0.24 M $CaCl_2$ and centrifuged once more. The supernatant was carefully decanted and the final pellet resuspended in 10 ml of culture medium (0.4 M sucrose, 1 x Gamborg B5 salts (Sigma), pH 5.8) and transferred to a 12 ml centrifuge tube. Viable protoplasts were purified and concentrated by centrifuging the tube at 150 *g* for 10 min. Living protoplasts floated as a tight band on top of the sucrose solution and were transferred to a clean 12 ml centrifuge tube with a 1 ml pipette. Protoplast viability and number were then determined before use.

2.15.2 Isolation of *Arabidopsis* leaf protoplasts

This protocol was obtained from Dr Jen Sheen (Department of Molecular Biology, Massachusetts General Hospital, Boston). *Arabidopsis* plants were germinated and grown, in low fluence rate of white light as described in 2.3.1, for 3-4 weeks until the leaves had fully expanded. Approximately 20 leaves were removed, piled together and cut into thin strips (1-2 mm) with a clean razor blade. A fresh blade was used after every 10 cuts. The leaf strips were then placed into a 9 cm Petri dish containing 20 ml enzyme solution (1% (w/v) cellulase R-10, 0.25% (w/v) macerozyme R-10, 0.4 M mannitol, 30 mM $CaCl_2$, 0.1% (w/v) BSA, 5 mM β-mercaptoethanol, 10 mM MES pH 5.7). This was done for about 10 Petri dishes worth of material. Petri dishes were then transferred to a vacuum chamber and a vacuum applied for 30 min. Digestion was

allowed to proceed, in darkness at room temperature, for 90 min with gentle shaking (40 rpm). After digestion of the cell wall, protoplasts were released by shaking at 80 rpm for 5 min. The digest was then filtered through a 100 µm steel sieve and the filtrate collected into 25 ml centrifuge tubes. Protoplasts were pelleted by centrifugation at 150 *g* for 2 min. The pellet was resuspended in 10 ml wash solution (0.4 M mannitol, 20 mM KCl, 4 mM MES pH 5.7) and recentrifuged. The final protoplast pellet was resuspended in 10 ml of culture medium (0.4 M sucrose, 1 x Gamborg B5 salts, pH 5.8) and purified and concentrated as described in 2.15.1. Protoplasts were counted and their viability determined before being used for transient expression studies.

2.15.3 Protoplast isolation from *Phaseolus vulgaris* primary leaves

This method was obtained from Dr Nigel Urwin (Division of Biochemistry and Molecular Biology, University of Glasgow). *Phaseolus vulgaris* plants were germinated and grown in white light as described in 2.3.1 for 14 days, until the primary leaves had fully expanded. Primary leaves (about 15) were removed and the upper surface rubbed lightly with alumina (Sigma, type A-5). The leaves were washed in several changes of dH_2O, floated upper surface down on 20 ml of enzyme solution (0.8 % (w/v) cellulase R10, 0.25 % (w/v) macerozyme R10, 0.4 M mannitol in CPW salts (27.2 mg l^{-1} KH_2PO_4, 101 mg l^{-1} KNO_3, 246 mg l^{-1} $MgSO_4$, 0.16 mg l^{-1} KI, 0.025 mg l^{-1} $CuSO_4$, 1.48 g l^{-1} $CaCl_2$), pH 5.8) in 9 cm Petri dishes. Digestion was allowed to proceed overnight in darkness at room temperature. Protoplasts were then released by gently rotating and tapping the Petri dish. Leaf debris was removed by filtration through a 70-80 µm steel seive into sterile 50 ml centrifuge tubes. Protoplasts were allowed to settle, on ice, for 1 hour and the supernatant gently removed with a 5 ml pipette. The pellet was resuspended in 1/2 volume of ice-cold culture medium (0.4 M mannitol in CPW salts, pH 5.8) and the protoplasts allowed to settle as before. This washing step was repeated once more and the final protoplast pellet resuspended in an

appropriate volume of culture medium before determining protoplast viability and concentration.

2.15.4 Determination of protoplast viability

Protoplast viability was determined using fluorescein diacetate (FDA). A stock solution of FDA (5 mg ml^{-1}) was prepared in acetone and stored in the dark at 4°C. Two drops of the FDA solution were added to 20 ml of the appropriate protoplast culture medium and mixed. Two hundred µl of this solution was mixed with an equal volume of the protoplast suspension and left at room temperature for 2 min. Protoplast viability was then determined using a fluorescence microscope (using blue illumination). Living protoplasts stained green whereas non-viable protoplasts stained red or remained unstained. The percentage viability in a random field of view could then be determined: (number of green fluorescing protoplasts/total number of protoplasts) x 100.

2.15.5 Counting protoplasts

Protoplasts were counted using a haemocytometer. A sample of the protoplast suspension (of a known volume) was introduced beneath the cover-slip, to fill the counting area of the haemocytometer. Using a microscope, the number of protoplasts enclosing the triple-lined square (enclosing 16 smaller squares) were counted (n), including those touching the top and left edges, but not the bottom or right edges. The number of protoplasts per ml was calculated as $5n \times 10^3$. Protoplasts were then diluted to the required concentration with the appropriate buffer.

2.16 Transient Gene Expression in Protoplasts

2.16.1 Polyethylene glycol-mediated DNA uptake

This method is a modified version of that obtained from Dr Bernd Weisshaar (Max-Planck-Institut, Köln, Germany). *Arabidopsis* protoplasts isolated as described in 2.15 were diluted to a concentration of 1 x 10^6 per ml with the appropriate buffer. Two hundred µl of the protoplast suspension was transferred to a round bottom 12 ml centrifuge tube and 20 µg of the appropriate plasmid DNA (in a volume of 40 µl) added. The suspension was then gently mixed by tapping/shaking the tube. Two hundred µl of polyethylene glycol (PEG) solution (25% (w/v) PEG 6,000 (Serva), 100 mM $Ca(NO_3)_2$ (Sigma), 45 mM mannitol, pH 9.0) was added, the solution mixed and left at room temperature for 15 min. After this period, 5 ml wash solution (275 mM $Ca(NO_3)_2$) was added and the tube centrifuged at 150 *g* for 5 min. The supernatant was decanted and the protoplasts gently resuspended in 6 ml culture medium (0.4 M sucrose, 1 x Gamborg B5 salts, pH 5.8). Transfections involving the same plasmid, were usually pooled together and mixed before each experiment in order to minimise variation in transfection efficiencies between samples. Experiments were carried out in duplicate. Protoplasts were separated into 3 ml aliquots in 12 ml UV-transmitting plastic centrifuge tubes then treated as described in chapter 5.

After treatment, transfected protoplasts were diluted with 3 volumes of 0.24 M $CaCl_2$. The protoplasts were mixed and pelleted at 150 *g* for 10 min. All but 1 ml of the supernatant was aspirated off and the pellet gently resuspended. The suspension was then transferred to a 1.5 ml Eppendorf® tube and centrifuged at 10,000 *g* for 30 s. The remaining supernatant was removed by aspiration and the protoplast pellet frozen in liquid nitrogen. Protoplasts were then stored at –80°C until use.

2.16.2 Electroporation

Phaseolus vulgaris protoplasts isolated as in 2.15.3 were resuspended in ice-cold electroporation buffer (0.4 M mannitol, 20 mM KCl, 4 mM MES pH 5.7) to give a protoplast concentration of 1 x 10^6 per ml. *Arabidopsis* protoplasts isolated as in 2.15.1 and 2.15.2 were diluted with 3 volumes of electroporation buffer and centrifuged at 150 *g* for 5 min. The protoplast pellet was then resuspended in ice-cold electroporation buffer to give a protoplast concentration of 1 x 10^6 per ml. Protoplasts were dispensed in 0.5 ml aliqouts into plastic 1 ml cuvettes and placed on ice for 30 min. Twenty five µl of plasmid DNA (in a volume of 30 µl) was added and the cuvette tapped to mix. Electrodes from the electroporation apparatus (IBI GeneZapper 450/2500, Kodak, USA) were inserted into the cuvette and the protoplasts electroporated under the appropriate conditions. Two aliquots (200 µl) of the protoplast solution (duplicate samples) were then transferred to the wells of a 12 x 3 ml tissue culture plate (Nunclon™) containing 0.8 ml of the desired culture medium. Transfected protoplasts were incubated under the appropriate light conditions as described in the results.

After incubation, protoplasts were transferred to a 1.5 ml Eppendorf® tube and centrifuged at 10,000 *g* for 1 minute. The supernatant was aspirated off and the protoplast pellet frozen in liquid nitogen. Protoplasts were then stored at –80°C until use.

2.17 Measurement of GUS Activity

Levels of β-glucuronidase (GUS) activity were measured by the method of Jefferson (1987) using 4-methyl umbelliferyl glucuronide (MUG) as substrate (Bio/Gene, Bolnhurst, UK). Transfected protoplasts harvested as described in 2.15.1 were thawed on ice before resuspending in 0.2 ml of GUS extraction buffer (50 mM Na_2PO_4 pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% (w/v) sarcosyl, 0.1% (w/v) Triton X-100). The protoplast suspension was vortexed thoroughly,

centrifuged at 10,000 *g* for 5 min, then returned to ice. One hundred µl of the soluble fraction was removed to a clean 1.5 ml Eppendorf® tube containing 100 µl of 2 mM MUG (dissolved in GUS extraction buffer) and inverted to mix. The remaining 100 µl of sample was kept for protein determination as described in 2.19. As a blank, a tube was prepared containing equal volumes (100 µl) of GUS extraction buffer and substrate. Tubes were then incubated at 37°C in a water bath. At intervals of 20, 40 and 60 min, the reaction was stopped by removing 40 µl from each tube to a 2 ml Eppendorf® tube containing 1.96 ml 0.2 M Na_2CO_3. These tubes were then mixed and the relative concentration of 4-methylumbelliferone (MU) production quantified using a spectrofluorimeter (Perkin Elmer LS5), excitation at 365 nm and emission at 455 nm. The 20, 40 and 60 min flourescence values for the blank were then subtracted from the values obtained for each sample to normalise against substrate decompostion at 37°C. GUS activity was calculated by calibrating the fluorimeter with solutions of MU (Sigma). A 100 mM stock solution of MU in 50% (v/v) EtOH was prepared and serially diluted 1/10 with GUS extraction buffer/0.2 M Na_2CO_3 (1/50) to obtain a series of standards (1, 10, 100, 1000 nM). The MU concentration in each sample was calculated using the standard curve of relative flourescence against MU concentration (nM). GUS activity was then calculated as pmole MU produced per min and normalised per mg protein as determined in 2.19.

2.18 Measurement of CAT Actvivity

Chloramphenicol acetyltransferase (CAT) activity was measured by the method of Seed and Sheen (1988). Transfected protoplasts harvested as described in 2.16.2 were thawed on ice and resuspended in CAT assay buffer (2mM $MgCl_2$, 20 mM Tris-HCl pH 8.0). The protoplast solution was vortexed thoroughly for 20 s and centrifuged at 10,000 *g* for 5 min. Fifty µl of the soluble fraction was transferred to a 1.5 ml Eppendorf® tube and an equal volume of reaction buffer (250 µM Tris-HCl pH 8.0, 500 µM butyryl-CoA, 200 µM chloramphenicol, 4 µCi [^{3}H]chloramphenicol (30-60 Ci

mmol^{-1}) added. As a blank, a tube was prepared containing equal volumes (50 µl) of CAT assay and reaction buffer. The reaction was incubated at 37°C for 2 hours and stopped by adding 2 volumes of mixed xylene isomers. The tube was then centrifuged at 10,000 *g* for 1 minute and the xylene layer (top phase) removed to a clean tube. An equal volume of TE (pH 8.0) was added and the tube centrifuged as above. This extraction procedure was repeated once more, to remove un-incorporated [^{3}H]chloramphenicol. The upper phase was then transferred to a scintillation vial containing 5 ml Ecoscint® and the radioactivity counted in a scintillation counter. Relative CAT activity was expressed as counts per minute (cpm). The cpm values for the blank were subtracted from the values obtained for each sample. *E. coli* CAT (Sigma) was used as a positive control for the assay.

2.19 Measurement of Protein

Protein levels were measured using the method of Bradford (1976) using BSA (Sigma) as standard. Bradford reagent was prepared by dissolving 100 mg of Coomassie brilliant blue G250 in 50 ml 95% (v/v) EtOH and 100 ml 85% (w/v) phosphoric acid. This was diluted to one litre with dH_2O and filtered through Whatman filter paper before use.

The protein sample of interest (5-10 µl) was diluted to 100 µl with dH_2O in a 1 ml cuvette. From a 1 mg ml^{-1} stock solution of BSA, 2, 4, 8, 16 and 32 µg standards were prepared by diluting to 100 µl with dH_2O in a 1 ml cuvette. One ml Bradford reagent was added to each cuvette, mixed by inversion and left at room temperature for 5 min. Levels of protein were determined by measuring the OD at 595 nm in a spectrophotometer. The protein standards were used to construct a standard curve of absorbance at 595 nm against amount of protein (µg). The amount of protein (µg) in each sample was then calculated.

2.20 Aequorin Studies

Transgenic *Arabidopsis* RLD seeds harbouring the aequorin gene driven by the cauliflower mosaic virus 35S promoter were supplied by Dr Janet Braam (Department of Biochemistry and Cell Biology, Rice University, Houston).

2.20.1 Growth of transgenic plants

Transgenic *Arabidopsis* seeds were surface-sterilised, germinated and grown as described in 2.3.1. After 14 days, plants were used for aequorin studies.

2.20.2 Reconstitution of aequorin

Two week old transgenic *Arabidopsis* plants, grown in agar medium, were transferred to 3 ml plastic cuvettes with a pair of tweezers. Plants were submerged in 2 μM coelenterazine (Cambridge Bioscience, UK) in 0.3% EtOH and incubated in the dark for 16 h. After incubation, plants were transferred to a fresh cuvette and kept in a low fluence rate of white light (<10 μmol m^{-2} s^{-1}) until use.

2.20.3 Measurement of aequorin activity

Aequorin luminescence measurements were made using a luminescence spectrophotometer (Perkin Elmer LS50), emission at 465 nm. Cold shock-induced luminescence was produced by injecting 2 ml ice-cold dH_2O onto a single plant in a plastic cuvette situated in the spectrophotometer. Cantharidin was also introduced by injection. Plants were illuminated in UV-transmitting plastic cuvettes with the light sources described in 2.4.1 for the times indicated and returned to the spectrophotometer.

Figure 2.1. Plasmid DNA used in this study

A number of plasmids were used throughout this study. The DNA and their source are described in the following table.

Plasmid	Description	Reference
pCHS	*Arabidopsis CHS* 1059 bp *Hind*III genomic DNA fragment in pUC19	Trezzini *et al.* (1993)
pPAL	*Arabidopsis PAL* 4.0 kb *Eco*RI/*Hind*III genomic fragment in pUC19	Trezzini *et al.* (1993)
pTCH3	*Arabidopsis TCH3* 650 bp *Eco*RI cDNA fragment in bluescript	Braam (1992)
pCAB	*Arabidopsis CAB* 1.65 kb *Eco*RI genomic fragment in pAB165	Leutwiler *et al.* (1986)
pH1	*Phaseolus vulgaris H1* 1.4 kb *Pst*I cDNA fragment in pAT153	Lawton and Lamb (1987)
pCaMVCN	*CaMV 35S* promoter-CAT fusion in pUC8	Obtained from Pharmacia (UK)
p35SGUS	*CaMV 35S* promoter-GUS fusion in pBT2	Obtained from Dr Bernd Weisshaar (Weisshaar *et al.*, 1991b)
p1969GUS	Full length *CHS* promoter-GUS fusion in pBT2	
p1448GUS	-1448 deletion *CHS* promter-GUS fusion in pBT2	
p672GUS	-672 deletion *CHS* promoter-GUS fusion in pBT2	
p336GUS	-336 deletion *CHS* promoter-GUS fusion in pBT2	
p164GUS	-164 deletion *CHS* promoter-GUS fusion in pBT2	
p94GUS	-94 deletion *CHS* promoter-GUS fusion in pBT2	
p62GUS	-62 deletion *CHS* promoter-GUS fusion in pBT2	
p4LRUGUS	Light response unit tetramer-GUS fusion in pBT2	

Figure 2.2. Spectra of the different light qualities used throughout this study

Six different light qualities were used in this study. The spectral photon distribution in each of the light conditions used was measured using a spectroradiometer (Macam SR9910) at the light intensity indicated.

(**A**) White (120 μmol m^{-2} s^{-1}).

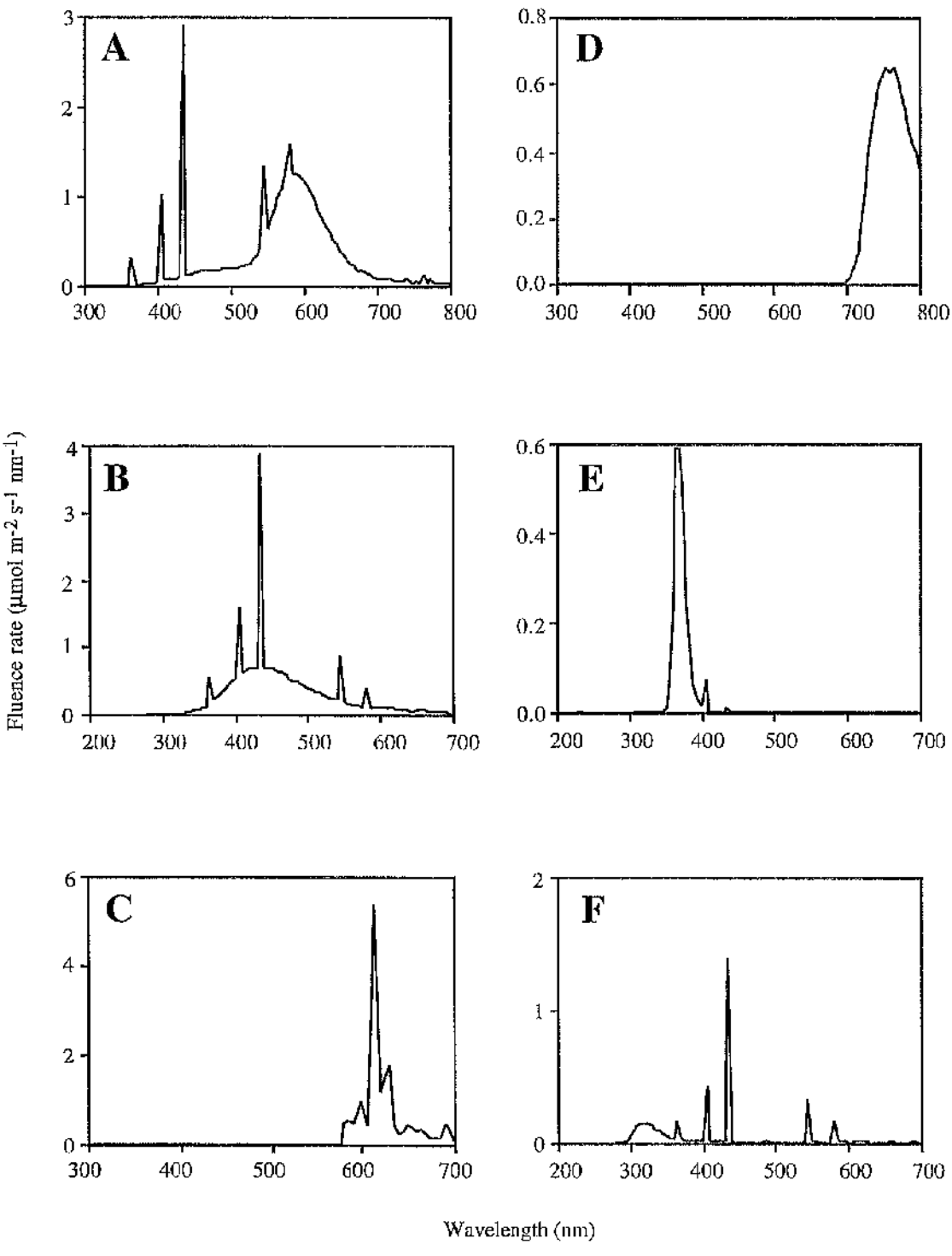
(**B**) UV-A/blue (80 μmol m^{-2} s^{-1}).

(**C**) Red (80 μmol m^{-2} s^{-1}).

(**D**) Far-red (45 μmol m^{-2} s^{-1}).

(**E**) UV-A (60 μmol m^{-2} s^{-1}).

(**F**) UV-B (3 μmol m^{-2} s^{-1}).


A
B
C
D
E
F
Fluence rate (µmol m-2 s-1 nm-1)
Wavelength (nm)

CHAPTER 3: *CHS* EXPRESSION IN *ARABIDOPSIS* CELL CULTURES

3.1 Introduction

To investigate the signal transduction pathways concerned with the UV-B and UV-A/blue light induction of *CHS* in *Arabidopsis* a cellular system was required which would permit biochemical experimentation. We chose to use an *Arabidopsis* cell suspension culture introduced by May and Leaver (1993). The aim of the experiments described in this chapter was to establish whether the *Arabidopsis* cell culture provides a suitable system for a biochemical and molecular dissection of the UV-B and UV-A/blue light signal transduction processes regulating *CHS* expression.

3.2 The *Arabidopsis* Cell Culture

The *Arabidopsis* cell culture was kindly donated by Dr Mike May (Department of Plant Sciences, University of Oxford). This green cell suspension was selected from calli of *Arabidopsis thaliana* cv Landsberg *erecta* and is shown in Figure 3.1A. The cell suspension, when viewed under the microscope, can be seen to consist of small aggregates of cells varying in shape and size (Fig. 3.1C). Transfer of cells to solid media results in the rapid growth of friable callus (Fig. 3.1B). The callus produced was maintained on solid media and could be used to initiate a new cell suspension in the event of contamination. Bacterial and fungal contamination of the cell culture was monitored periodically (every 2-3 months) as described in 2.3.3. Subculturing of cells was carried out using a sterile plastic pipette. By this means, small cell aggregates and single cells were effectively selected.

3.3 Growth of the *Arabidopsis* Cell Culture

The cell suspension was previously grown in a light/dark cycle at 26°C (May and Leaver, 1993). In our controlled environment rooms, the cell culture was grown at 20°C in a continuous low fluence rate of white light. Therefore, prior to gene expression studies, it was important to determine the rate of cell growth under these conditions. This is shown in Figure 3.2. Despite an initial lag phase, the cell suspension enters a rapid growth phase 2 days after subculture and reaches a plateau after about 6 days. Thus, for all experiments, unless otherwise stated, cells were used on the third day after subculture (the point at which cells have entered exponential growth phase).

May and Leaver (1993) described the cell suspension as being heterotrophic i.e. dependent solely on organic matter for growth. However, it was observed that both light and a carbon source were required for maximal growth of the cell culture. Removal of either light or sucrose caused cell growth to diminish and eventually, at least after one week, led to cell death. As a result, the cell suspension was considered to be photomixotrophic.

3.4 UV-B and UV-A/blue Light Regulate *CHS* Transcript Accumulation in the *Arabidopsis* Cell Culture

It was important to initially establish whether the photoregulation of *CHS* expression in the *Arabidopsis* cell culture was similar to that observed in the intact plant. *Arabidopsis* plants were grown in a low fluence rate of white light for 21 days and placed in the dark for a further 2 days. Plants were then transferred to different light qualities for 16 h, total leaf RNA isolated and *CHS* transcripts analysed. As shown in Figure 3.3A, *CHS* transcripts are not detectable in dark-adapted *Arabidopsis* leaf tissue. Similarly, *CHS* transcripts are at a very low level in plants transferred to a low fluence rate of white light but show a large increase following transfer to a 6-fold higher fluence rate. Transfer of plants to UV-B or UV-A/blue light also induces *CHS* transcript

accumulation, but red light does not. Figure 3.3B demonstrates that the differential light induction of *CHS* transcripts is not due to unequal loading of total RNA. These results are consistent with previous studies that indicate that *CHS* expression in light-grown *Arabidopsis* is induced by UV-B and UV-A/blue light and high intensity white light (Feinbaum and Ausubel, 1988; Feinbaum *et al.*, 1991; Kubasek *et al.*, 1992; Li *et al.*, 1993; Jackson *et al.*, 1995; Batschauer *et al.*, 1996). The lack of induction by red light is consistent with the observation that phytochrome has little effect on *CHS* expression except in young *Arabidopsis* seedlings (Kaiser *et al.*, 1995).

The *Arabidopsis* cell culture was routinely grown in a low fluence rate of white light which induces a very low level of *CHS* transcipts in mature plants (Fig. 3.3A). The cells were transferred to different light qualities for 24 h, total RNA extracted and *CHS* transcripts analysed. As shown in Figure 3.3C, *CHS* transcripts are at a very low level in *Arabidopsis* cells grown in a low fluence of white light. Transfer of the culture to high intensity white light, UV-A/blue light or UV-B induces *CHS* transcript accumulation whereas red or far-red light was without effect. A probe encoding the *H1* gene product (Lawton and Lamb, 1987), which appears to be constitutively expressed in plant cells, was used as a control. None of the light treatments had any inductive effect on the level of *H1* transcripts, indicating their effects on *CHS* transcript accumulation did not result from a general induction of gene expression. Thus the regulation of *CHS* gene expression by different light qualities in the *Arabidopsis* cell culture is very similar to that observed in light-grown *Arabidopsis*.

3.5 UV-B Induction is Specific to Wavelengths Below 320 nm

Figure 2.2F illustrates that the UV-B source also emits very low levels of UV-A and blue light. Therefore, prior to further studies, we investigated whether these wavelengths were involved in regulating *CHS* expression in the *Arabidopsis* cell culture. For this experiment, cells were irradiated with either the standard UV-B source or with a UV-B source covered with a filter that prevented the transmission of

wavelengths below 320 nm. Cells were harvested after 4 and 8 h, total RNA isolated and *CHS* transcripts analysed. Figure 3.4 demonstrates that the effect of the UV-B source on *CHS* expression is specific to wavelengths below 320 nm because removal of these wavelengths prevents transcript accumulation. *H1* transcript levels were unaffected by either light treatment.

3.6 Kinetics of *CHS* Induction in Response to Different Light Qualities

Figure 3.5 shows the induction kinetics of *CHS* expression in *Arabidopsis* cells irradiated with UV-B, UV-A/blue light and high intensity white light for 24 h. After exposure to UV-B and UV-A/blue light, *CHS* transcript accumulation is detectable within 4 h and reaches a steady state after 12 h. In high intensity white light, *CHS* expression is observed after 2 h and is again not transient over a 24 h period. In each case, no change in *H1* transcript levels was observed over the duration of the time course.

3.7 Effects of a Simultaneous UV-B and UV-A/blue light Treatment on *CHS* Expression

Recent studies show that UV-A and blue light act synergistically with UV-B to enhance *CHS* transcript accumulation and *CHS* promoter activity in light-grown *Arabidopsis* (G. Fuglevand, J.A. Jackson and G.I. Jenkins, unpublished). Consistent with these findings, Figure 3.6A shows that illumination of light-grown *Arabidopsis* plants with UV-B and UV-A/blue light together results in a much greater induction of *CHS* transcript accumulation than with UV-A, UV-B and UV-A/blue light alone. The effect of UV-B and UV-A/blue light given together is more than additive and the response is therefore said to be synergistic. Figure 3.6B demonstrates that the induction of *CHS* transcripts in response to differential light qualities is not due to unequal loading of total

RNA. It was then investigated whether a similar pattern of expression could be observed in the *Arabidopsis* cell culture. In contrast to the situation found in plants, illumination of cells with UV-B and UV-A/blue light together gave an additive rather than a synergistic response (Fig. 3.6C). A simultaneous UV-B and UV-A/blue light treatment had no effect on the level of *H1* transcripts. This implies that the *Arabidopsis* cell culture does not show all aspects of *CHS* regulation observed in light-grown plants.

3.8 Low Temperature Induces *CHS* Expression

The possibility of using the cell culture for studying other signal transduction pathways regulating *CHS* was investigated. Since flavonoid biosynthesis genes, including *CHS*, are induced by low temperature (Christie *et al.*, 1994; Leyva *et al.*, 1995), the effect of a cold treatment on *CHS* expression in *Arabidopsis* cells was examined. Cells growing in a low fluence rate of white light at 20°C were transferred to a cold room (8°C) with similar light conditions. After 1 and 2 days, cells were harvested, total RNA isolated and *CHS* transcripts analysed. As shown in Figure 3.7, cold treatment stimulates *CHS* transcript accumulation in the *Arabidopsis* cell culture. Consistent with the expression pattern found in light-grown *Arabidopsis* (Leyva *et al.*, 1995), *CHS* transcripts are strongly induced within 24 h and still detectable after 2 days. No increase in *CHS* transcripts was observed in cells grown in an equal fluence rate of white light at 20°C for 24 h. Exposure of the culture to low temperature had no effect on *H1* transcript levels. Thus *CHS* gene expression in the *Arabidopsis* cell culture is cold-regulated in a very similar manner to that observed in whole plants.

3.9 Effect of Jasmonic Acid on *CHS* Expression

Jasmonic acid and its volatile methyl ester have been implicated in plant defense responses to wounding and pathogen attack (Dixon *et al.*, 1994). Recent studies have

shown that methyl jasmonate stimulates the expression of flavonoid biosynthesis genes, including *CHS*, in detached petunia corollas (Tamari *et al.*, 1995). Whether jasmonic acid could induce *CHS* expression in the *Arabidopsis* cell culture was therefore examined. Figure 3.8 shows that incubation of cells with jasmonic acid, at concentrations effective in other systems (Creelman *et al.*, 1992; Tamari *et al.*, 1995), failed to increase the *CHS* transcript level above that normally present in low white light. On the other hand, jasmonic acid induced *PAL* gene expression, demonstrating the lack of effect on *CHS* was not due to a lack of uptake. Levels of *H1* transcripts were unaffected by the jasmonic acid treatment. Therefore, in constrast to other plant systems (Dixon *et al.*, 1994), jasmonic acid does not appear to regulate *CHS* gene expression in the *Arabidopsis* cell culture.

3.10 *CHS* Expression in an *Arabidopsis hy4* Cell Culture

The above findings demonstrate that the *Arabidopsis* cell culture provides a model system of reduced complexity for studying the regulation of gene expression. We investigated whether this approach could be extended to mutant backgrounds, by establishing a cell suspension culture from the CRY1-deficient mutant, *hy4-2.23N* (Koorneef *et al.*, 1980). Using culture conditions similar to those described by May and Leaver (1993), well developed calli with a pale-yellow colour were obtained within one month. In our hands however, 0.5 mg l^{-1} NAA produced hard, rooty callus whereas an equivalent concentration of 2,4-D induced the formation of callus which was soft and friable. Similar concentrations of this synthetic auxin have been used to establish friable callus from *Arabidopsis* seedlings (Axelos *et al.*, 1992; Doelling and Pikaard, 1993; Trezzini *et al.*, 1993). When inoculated into liquid medium and placed on a rotary shaker, the friable clumps dissociate to produce a primary cell suspension. After a subculture period of about 3 months, a chunky, yellow cell suspension was obtained as shown in Figure 3.9A. This culture was used for gene expression studies. Cells, routinely grown in a low fluence rate of white light, were transferred to different

light qualities for 6 h, total RNA isolated and *CHS* transcripts analysed. Once again, the probe encoding the *H1* gene product was used as a control. As shown in Figure 3.9B, *hy4* cells are strongly impaired in the induction of *CHS* transcripts in UV-A/blue light, similar to *hy4* leaf tissue (Jackson and Jenkins, 1995). However, no difference was observed between the *hy4* and wild-type cultures in the level of *CHS* transcripts induced by UV-B, which is again consistent with the *CHS* expression pattern found in mature *hy4* leaves (G. Fuglevand, J.A. Jackson and G.I. Jenkins, unpublished). Thus these results not only show that the UV-B induction of *CHS* is independent of CRY1 but demonstrate the feasibility of establishing mutant cell cultures for biochemical experimentation.

3.11 Discussion

In this chapter, the possibility of using a photomixotrophic cell suspension from *Arabidopsis* (May and Leaver, 1993) as a model system for studying signal transduction events concerned with the UV-B and UV-A/blue light induction of *CHS* expression was explored. The results not only show that the cell culture provides a suitable system to study gene expression in response to UV-B, UV-A/blue light and other stimuli, but demonstrate that the approach can be extended to mutant backgrounds.

3.11.1 Analysis of gene expression

Throughout this study gene expression was measured by analysing the transcript levels of several genes. In each experiment, only one size of *CHS* transcript was detected. From the 25S and 18S ribosomal RNA sizes it was calculated that the *Arabidopsis* cDNA probe hybridised to a trancript with an approximate size of 1400 nucleotides. This is consistent with the size of *CHS* transcripts found by Feinbaum and Ausubel (1988). However, in some experiments the *Arabidopsis CHS*, *PAL*, *CAB* and

Phaseolus vulgaris H1 cDNA probes hybridised to a second or third transcript. The positions of these extra transcripts were found to correspond to the 25S and 18S ribosomal RNA bands, implying that they were most likely due to non-specific hybridisation.

In all experiments involving the *Arabidopsis* cell culture, a probe encoding the *H1* gene product (Lawton and Lamb, 1987) was used as a control. *H1* transcript levels were unaffected by the various treatments used in each experiment, demonstrating the effects observed on *CHS*, *CAB* and *PAL* transcripts were not due to a general up-regulation of gene expression.

Unless otherwise stated, the experiments described in this study were repeated three to six times and in each case similar trends were observed. The data presented are from individual experiments which are representative of the results obtained.

3.11.2 The *Arabidopsis* cell culture provides a model system to study the UV-B and UV-A/blue light regulation of *CHS*

The photomixotrophic *Arabidopsis* cell culture used here is similar to the soybean cell culture that has provided information on the signalling processes coupling phytochrome to *CAB*, *rbcS*, *fnr* and *CHS* gene expression (Lam *et al.*, 1989a; Romero and Lam, 1993; Bowler *et al.*, 1994a, 1994b). However, in contrast to the soybean system, *CHS* is not regulated by phytochrome in the *Arabidopsis* cell culture, as shown by the lack of induction by either red or far-red light (Fig. 3.3C). This is consistent with the lack of phytochrome induction of *CHS* in all but the youngest *Arabidopsis* seedlings (Feinbaum *et al.*, 1991; Kubasek *et al.*, 1992; Kaiser *et al.*, 1995). These findings suggest a developmental switch in photoreceptor usage in the light regulation of *CHS* expression in *Arabidopsis*. Similar developmental changes in the induction of *CHS* expression have been described for mustard and parsley (Batschauer *et al.*, 1991; Frohnmeyer *et al.*, 1992). Furthermore, the regulation of *CHS* expression in the *Arabidopsis* cell culture by UV-B, UV-A/blue light and high intensity white light is

similar to the situation in mature *Arabidopsis* leaf tissue (Fig. 3.3A; Li *et al.*, 1993; Jackson *et al.*, 1995; G. Fuglevand and G.I. Jenkins, unpublished). Only a low fluence rate of UV-B, specific to wavelengths below 320 nm and similar to the levels found in sunlight, is required to induce *CHS* (Fig. 3.4). The *Arabidopsis* cell culture therefore provides an excellent, homogeneous cellular system for the biochemical and molecular dissection of the UV-B and UV-A/blue light signalling pathways regulating *CHS* expression. Moreover, the rapidity of the response facilitates biochemical investigation of the signal transduction processes involved (Fig. 3.5).

3.11.3 Distinct UV-B and UV-A/blue phototransduction pathways regulate *CHS*

An important feature of *Arabidopsis* is that desired mutant phenotypes can be induced relatively easily (Meyerowitz, 1989). The development of the CRY1-deficient, *hy4* cell culture demonstrates that the cellular approach can be extended to mutant backgrounds. Moreover, the *hy4* cell suspension may be useful for studying CRY1 signal transduction processes. Indeed, microinjection studies with the phytochrome-deficient *aurea* mutant of tomato have been instrumental in defining components of the phytochrome signalling pathways (Neuhaus *et al.*, 1993). The fact that the *hy4* cell culture is impaired in the induction of *CHS* by UV-A/blue light demonstrates that CRY1 mediates the effects of these wavelengths (Fig. 3.9), which is consistent with the absorption properties of the flavin chromophore bound to CRY1 in insect cells (Lin *et al.*, 1995a). UV-B induction of *CHS* expression is unaltered in *hy4* cells, indicating that the CRY1 photoreceptor is not involved in this response. Therefore, distinct UV-B and UV-A/blue light signalling pathways concerned with the induction of *CHS* expression can be identified. These findings are consistent with the *CHS* expression pattern found in *hy4* plants (Ahmad *et al.*, 1995; Jackson and Jenkins, 1995; G. Fuglevand, J.A. Jackson and G.I. Jenkins, unpublished). Studies have shown that *hy4* plants have a reduced level of *CHS* transcripts relative to the wild-type in white

light (Chory, 1992; Jackson and Jenkins, 1995). The strong elevation of *CHS* transcripts by increasing fluence rates of white light in the wild-type is likely to be due to the increasing amount of blue light but may also be due to light stress at very high fluence rates. No reduction in *CHS* transcripts in response to white light was observed in the *hy4* cell culture (Fig. 3.9). However, the extremely high fluence rate used in this experiment makes it difficult to draw any real conclusions regarding this difference.

3.11.4 Synergistic UV-A/blue pathways do not appear to regulate *CHS* in the *Arabidopsis* cell culture

Recent studies have revealed a considerable degree of complexity, both in photoreception and signal transduction, in the UV/blue light regulation of *CHS* in *Arabidopsis* leaf tissue (G. Fuglevand, J.A. Jackson and G.I. Jenkins, unpublished). The data provide evidence for separate 'inductive' and 'synergistic' UV/blue phototransduction pathways regulating *CHS* promoter function and transcript accumulation. As in the *Arabidopsis* cell culture, at least two distinct pathways, a UV-A/blue light pathway involving CRY1 and a UV-B pathway, induce *CHS* expression in mature *Arabidopsis*. Furthermore, in leaf tissue, there appear to be distinct blue and UV-A pathways which interact synergistically with the UV-B pathway to provide maximal levels of expression (Fig. 3.6A). The UV-A and blue synergistic pathways appear to be distinct as they produce transient and relatively stable 'signals' respectively. Neither of these pathways appear to involve CRY1. Such complex interactions would allow for a greater sensitivity of *CHS* expression, and hence flavonoid production in response to UV/blue light. In the natural environment, plants are not exposed to UV-B light in the absence of UV-A/blue light. Thus this mechanism may provide a selective advantage against the damaging effects of UV-B irradiation. Nevertheless, we failed to observe any synergistic interaction between UV-B and UV-A/blue light the *Arabidopsis* cell culture (Fig. 3.6C). Whether the blue and UV-A synergistic pathways are inactive in the *Arabidopsis* cell culture is unknown. It is

possible that these pathways are undeveloped in the *Arabidopsis* cell culture. This does not appear to be a general feature of cultured plant cells as blue light has been reported to potentiate a subsequent increase in the UV-B induction of *CHS* expression in parsley cells (Ohl *et al.*, 1989).

3.11.5 Induction of *CHS* in response to other stimuli

In addition to light, *CHS* expression is stimulated by an array of environmental and endogenous signals (Dangl *et al.*, 1989). However, the regulation of *CHS* is strongly dependent on the plant species tested. For example, fungal pathogens induce *CHS* expression in legumes, such as soybean, but not in parsley (Hahlbrock and Scheel, 1989). In light-grown *Arabidopsis*, low temperatures have been shown to induce *CHS* transcript accumulation in a light-dependent manner (Leyva *et al.*, 1995). Similarly, *Arabidopsis* cells retain the ability to respond to low temperatures with respect to *CHS* expression (Fig. 3.7). The *Arabidopsis* cell culture may therefore be useful in characterising this response.

Treatment of cells with jasmonic acid was insufficient to increase the *CHS* transcript level in low white light (Fig. 3.8). The increase in *PAL* transcripts in the same experiment provides good evidence that the lack of effect on *CHS* expression was not due to a lack of uptake. Jasmonic acid has previously been shown to stimulate *PAL* transcript accumulation in soybean cells (Gundlach *et al.* 1992) and is proposed to function as a mediator of plant defense against pathogen attack. However, jasmonic acid has also been implicated in plant responses to wounding (Creelman *et al.*, 1992; Dixon *et al.*, 1994; Tamari *et al.*, 1995). Nevertheless, these findings indicate that the *Arabidopsis* cell culture system may be suitable for studying the cellular aspects of responses to other stimuli, such as pathogen invasion or wounding.

The cell culture therefore appears to be a versatile model system for studies of signal transduction in plant cells.

Figure 3.1. Photographs of the *Arabidopsis* cell and callus cultures

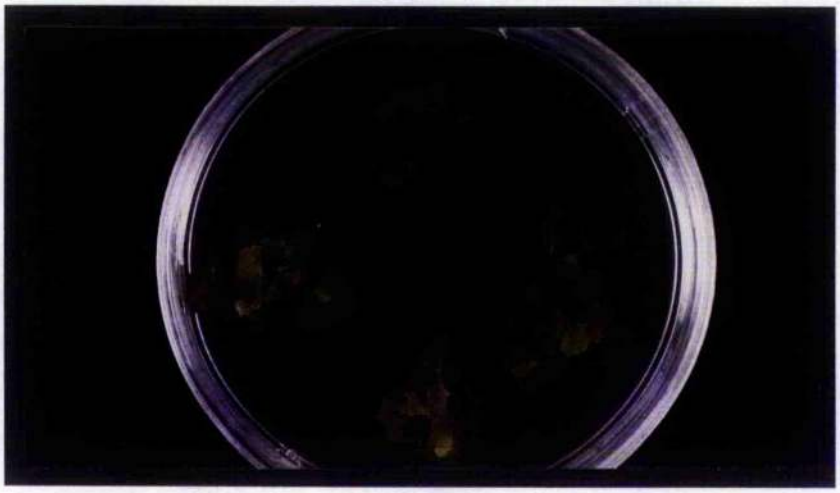
(A) A five day old *Arabidopsis* cell suspension grown in a 500 ml flask at 20°C in continuous 20 μmol m^{-2} s^{-1} white light.

(B) *Arabidopsis* callus growing on solid medium in a Petri dish.

(C) The same suspension culture cells as in (A) viewed under a microscope. The size bar represents approximately 60 μm.

A

B



C

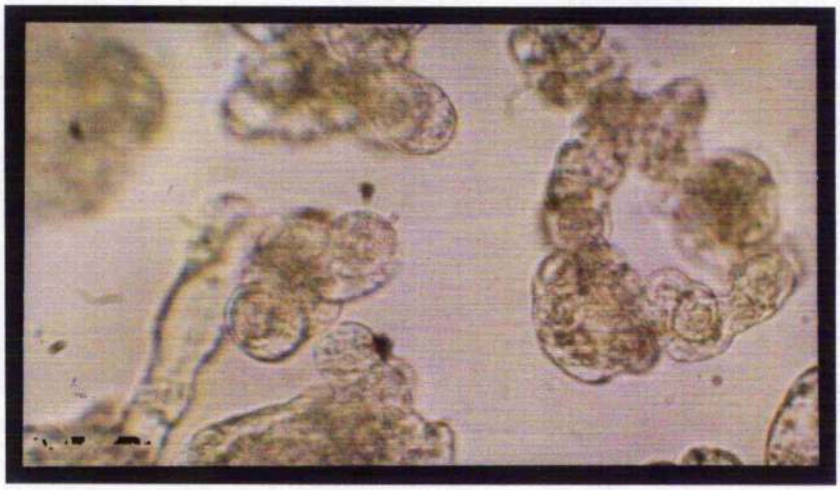


Figure 3.2. Growth curve of the *Arabidopsis* cell culture

Cells were grown in 20 μmol m^{-2} s^{-1} white light for 7 days after subculture with constant shaking. Aliquots of cells (10 ml) were harvested at the times indicated and the fresh weight (g l^{-1}) determined after drying the cell aliquot by vacuum filtration.

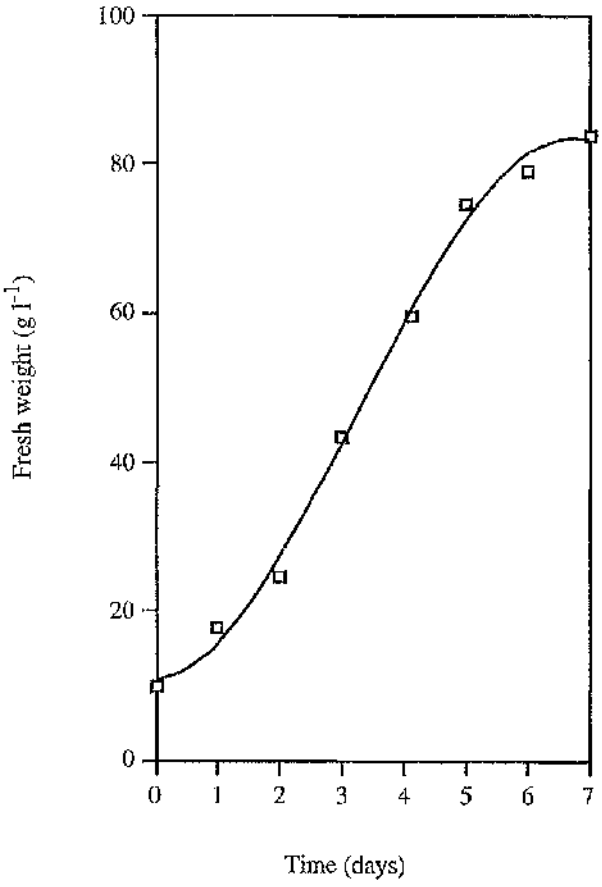

100
80
60
40
20
0
Fresh weight (g l-1)
0
1
2
3
4
5
6
7
Time (days)

Figure 3.3. UV-B and UV-A/blue light induce *CHS* transcript accumulation in the *Arabidopsis* cell culture

(A) *Arabidopsis* plants were grown in 20 μmol m^{-2} s^{-1} white light for 21 days, dark-adapted for 2 days then transferred to the following light qualities for 16 h: dark (lane1), 20 μmol m^{-2} s^{-1} white (lane 2), 120 μmol m^{-2} s^{-1} white (lane 3), 80 μmol m^{-2} s^{-1} red (lane 4), 80 μmol m^{-2} s^{-1} UV-A/blue (lane 5) or 3 μmol m^{-2} s^{-1} UV-B (lane 6). Total RNA was isolated from leaves, 10 μg separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. *CHS* transcript levels were analysed using an *Arabidopsis CHS* probe. The membrane was then washed and subjected to autoradiography.

(B) Photograph of the EtBr-stained gel before blotting. Lanes 1 to 6 correspond to those shown in (A).

(C) Cells grown in 20 μmol m^{-2} s^{-1} white light were transferred to the following different light qualities for 24 h: 20 μmol m^{-2} s^{-1} white (lane 1), 120 μmol m^{-2} s^{-1} white (lane 2), 80 μmol m^{-2} s^{-1} red (lane 3), 45 μmol m^{-2} s^{-1} far-red (lane 4), 80 μmol m^{-2} s^{-1} UV-A/blue (lane 5) or 3 μmol m^{-2} s^{-1} UV-B (lane 6). *CHS* transcripts were then analysed as described in (A). As a loading control, the membrane was stripped of radioactivity and reprobed with a cDNA encoding the constitutively expressed *H1* gene product from *Phaseolus vulgaris*.

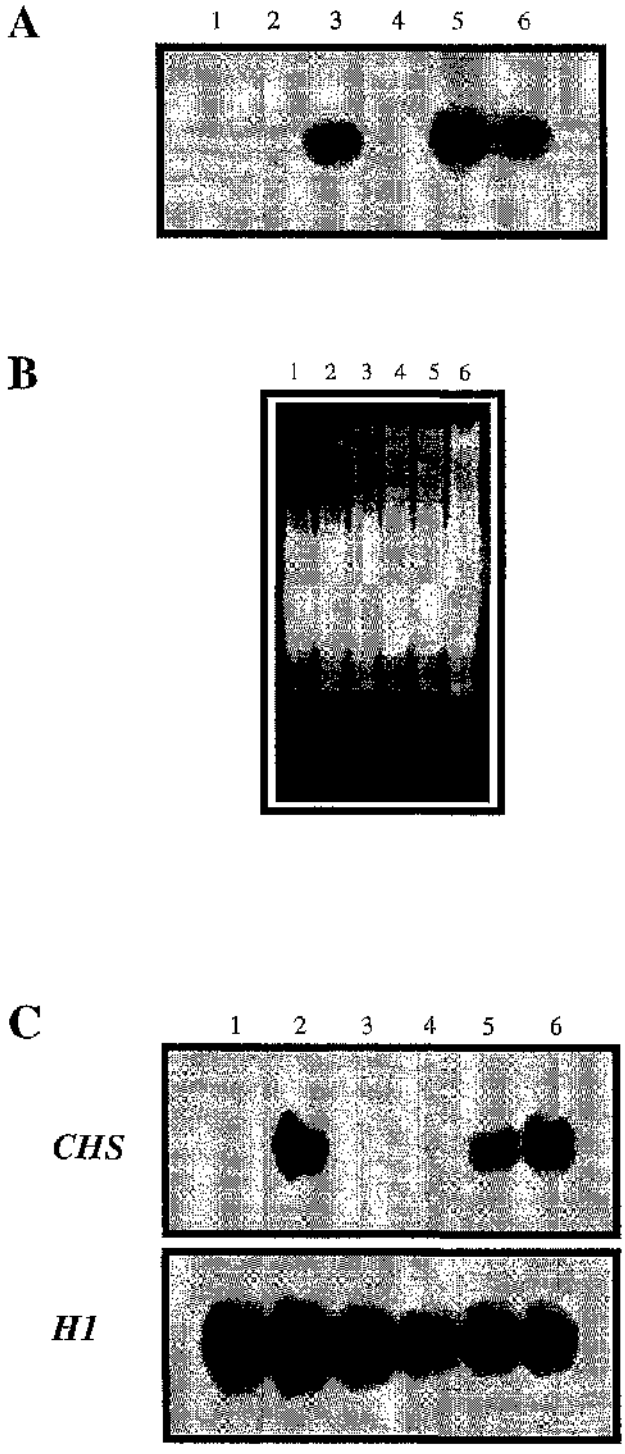

A
1 2 3 4 5 6
B
1 2 3 4 5 6
C
1 2 3 4 5 6
CHS
H1

Figure 3.4. UV-B induction of *CHS* is specific to wavelengths below 320 nm

Cells grown in 20 μmol m^{-2} s^{-1} white light (lane 1) were either illuminated with the standard 3 μmol m^{-2} s^{-1} UV-B source for 4 hours (lane 2) and 8 hours (lane 3) or with the UV-B source covered in a filter to prevent the transmission of wavelengths below 320 nm for 4 hours (lane 4) and 8 hours (lane 5). Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. *CHS* transcript levels were measured using an *Arabidopsis CHS* probe, the membrane washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and rehybridised to a *Phaseolus vulgaris H1* probe.

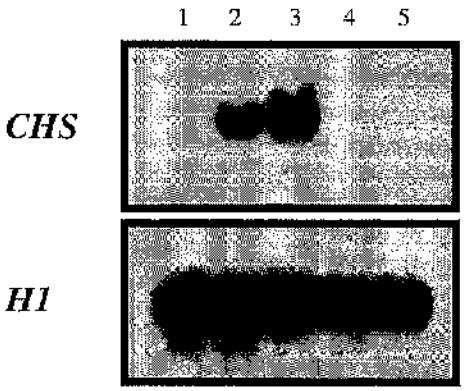

1 2 3 4 5
CHS
H1

Figure 3.5. Time course of *CHS* induction in response to different light qualities

Cells grown in 20 μmol m^{-2} s^{-1} white light were transferred to **(A)** 3 μmol m^{-2} s^{-1} UV-B, **(B)** 80 μmol m^{-2} s^{-1} UV-A/blue light or **(C)** 120 μmol m^{-2} s^{-1} white light for the times indicated. Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The membrane was probed with an *Arabidopsis CHS* probe, washed and autoradiographed. The membrane was then stripped of radioactivity and rehybridised to a *Phaseolus vulgaris H1* probe.

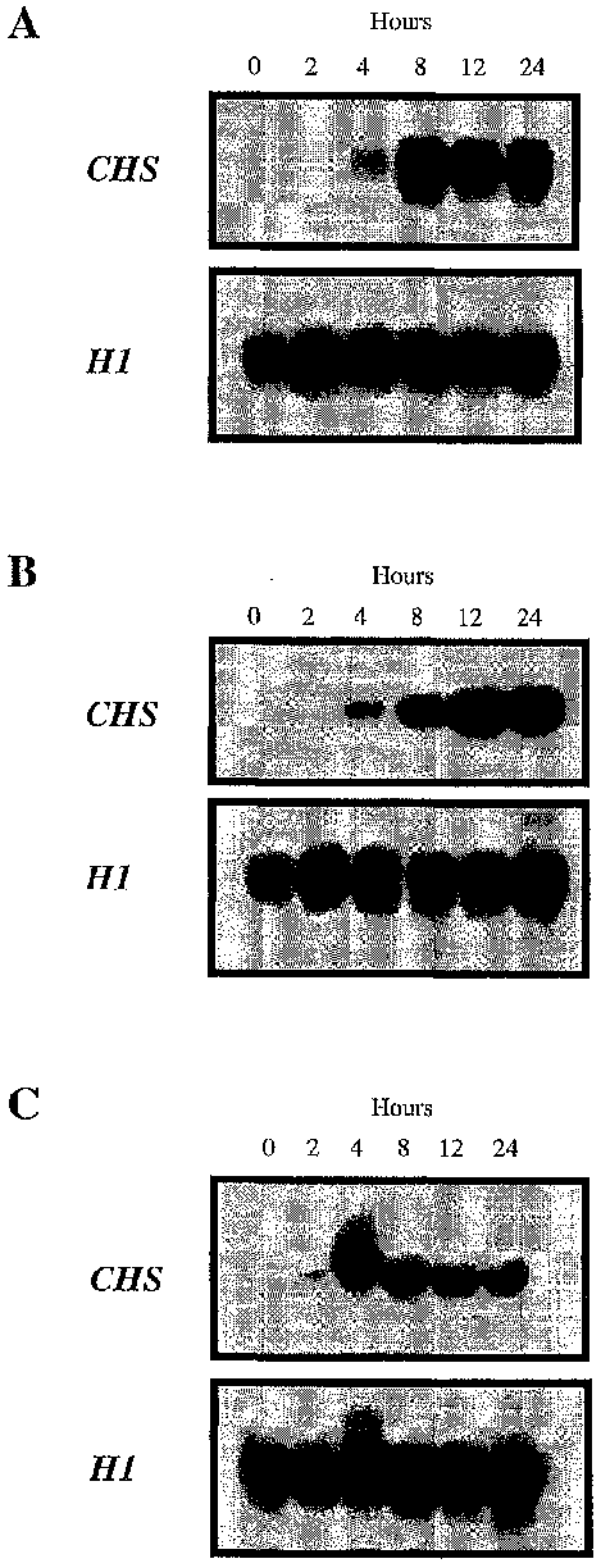

A
Hours
0 2 4 8 12 24
CHS
H1
B
Hours
0 2 4 8 12 24
CHS
H1
C
Hours
0 2 4 8 12 24
CHS
H1

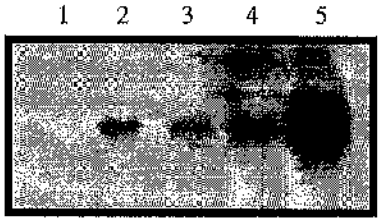
Figure 3.6. Effects of a simultaneous UV-B and UV-A/blue light treatment on *CHS* transcript accumulation

(A) *Arabidopsis* plants grown in 20 μmol m^{-2} s^{-1} white light for 21 days were transferred to the following light qualities for 8 h: 20 μmol m^{-2} s^{-1} white (lane 1), 60 μmol m^{-2} s^{-1} UV-A (lane 2), 3 μmol m^{-2} s^{-1} UV-B (lane 3), 80 μmol m^{-2} s^{-1} UV-A/blue (lane 4) or 3 μmol m^{-2} s^{-1} UV-B plus 80 μmol m^{-2} s^{-1} UV-A/blue (lane 5). RNA was isolated from leaves, 10 μg separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and autoradiographed.

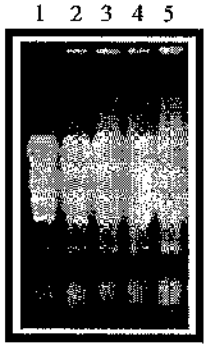
(B) Photograph of the EtBr-stained gel before blotting. Lanes 1 to 5 correspond to those shown in (A).

(C) Cells grown in 20 μmol m^{-2} s^{-1} white light were transferred to the following light conditions for 8 h: 20 μmol m^{-2} s^{-1} white (lane 1), 3 μmol m^{-2} s^{-1} UV-B (lane 2), 80 μmol m^{-2} s^{-1} UV-A/blue (lane 3) or 3 μmol m^{-2} s^{-1} UV-B plus 80 μmol m^{-2} s^{-1} UV-A/blue (lane 4). *CHS* transcripts were analysed as described in (A). The membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

A



B



C

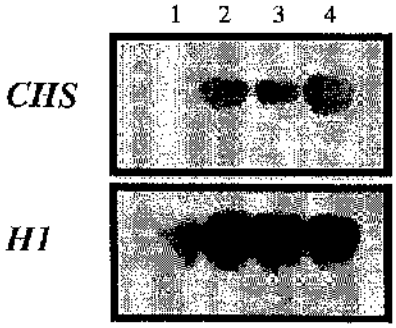


Figure 3.7. Low temperature increases *CHS* transcript levels

Cells grown at 20°C in 20 μmol m^{-2} s^{-1} white light (lane 1) were either kept in these conditions for 24 h (lane 2) or transferred to a cold room (8°C) with the same light conditions for 24 h (lane 3) or 48 h (lane 4). Total RNA was then isolated, 20 μg separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. The membrane was then stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

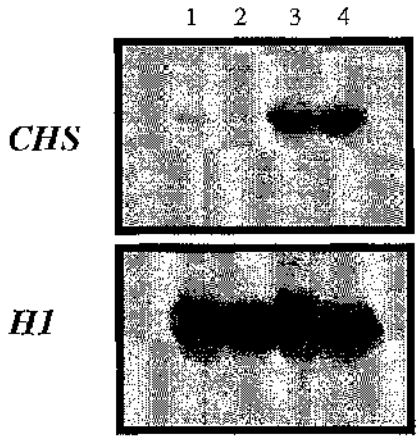

1 2 3 4
CHS
H1

Figure 3.8. Jasmonic acid increases *PAL* but not *CHS* transcript levels

Cells grown in 20 μmol m^{-2} s^{-1} white light (lane 1) were treated either without (lane 4) or with 10 μM jasmonic acid (lane 2) and 50 μM jasmonic acid (lane 3) for 20 h in 20 μmol m^{-2} s^{-1} white light After treatment, RNA was extracted, 10 μg separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis PAL* probe then a *Phaseolus vulgaris H1* probe.

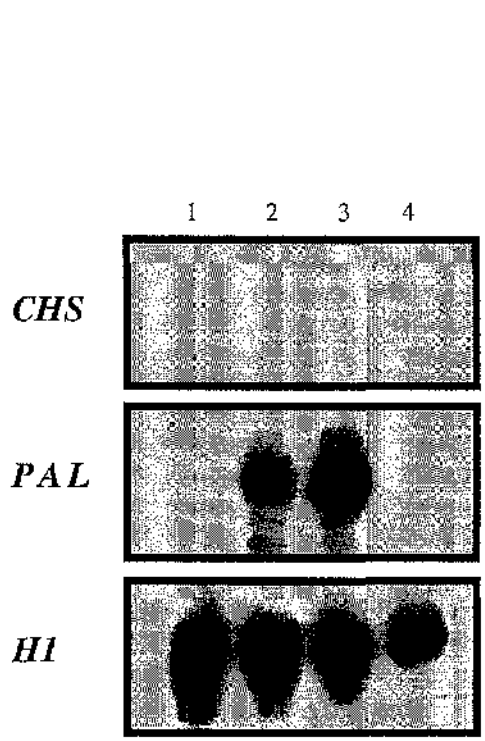
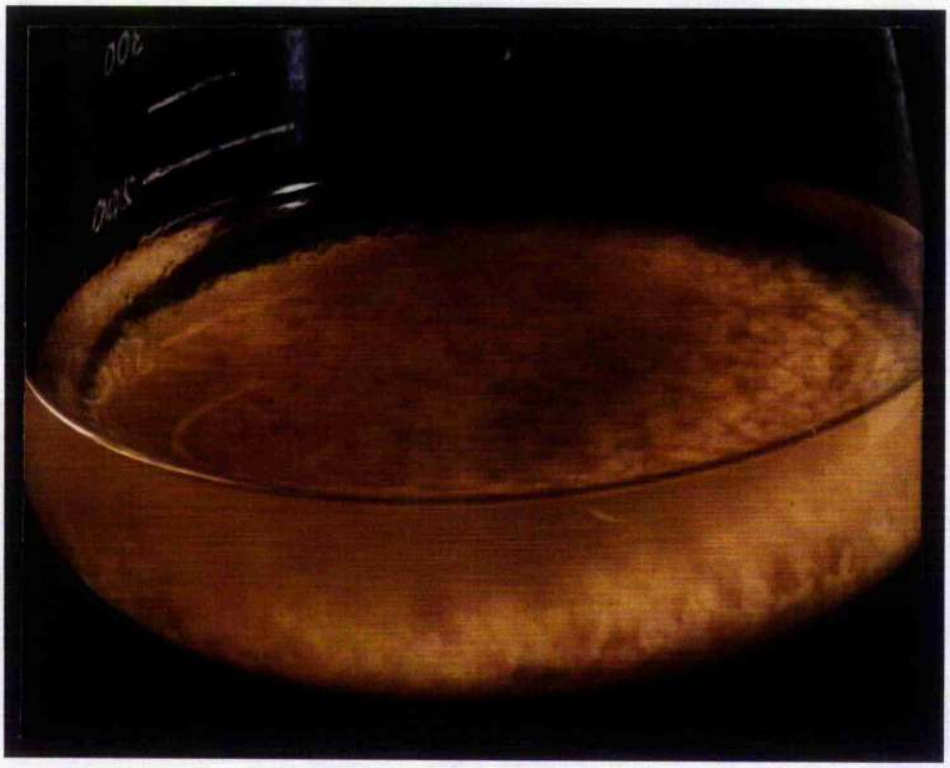

1
2
3
4
CHS
PAL
H1

Figure 3.9. *CHS* transcript accumulation in a *hy4* cell culture

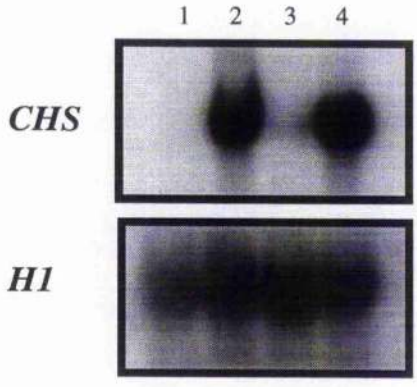
(A) Photograph of the *hy4* cell culture grown in a 500 ml flask.

(B) *hy4* cells grown in 20 μmol m^{-2} s^{-1} white light were transferred to the following different light qualities for 20 h: 20 μmol m^{-2} s^{-1} white light (lane 1), 200 μmol m^{-2} s^{-1} white light (lane 2), 80 μmol m^{-2} s^{-1} UV-A/blue light (lane 3) or 3 μmol m^{-2} s^{-1} UV-B (lane 4). Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. *CHS* transcript levels were measured using an *Arabidopsis CHS* probe and the membrane stripped of radioactivity before rehybridising to a *Phaseolus vulgaris H1* probe.

A



B



CHAPTER 4: UV-B AND UV-A/BLUE LIGHT SIGNAL TRANSDUCTION REGULATING *CHS*

4.1 Introduction

In the present chapter, a pharmacological approach was used in the *Arabidopsis* cell suspension culture to identify likely signal transduction processes concerned with the induction of *CHS* expression by UV-B and UV-A/blue light. The results demonstrate that a dissection of the UV-B and UV-A/blue phototransduction pathways regulating *CHS* is experimentally feasible with the cell culture system. Furthermore, the data show that the signalling pathways are complex and provide initial information on their components.

4.2 Requirement for Calcium in the UV-B and UV-A/blue Light Regulation of *CHS*

Calcium is ubiquitously involved in a variety of plant responses (Johannes *et al.*, 1991; Bush, 1993; Poovaiah and Reddy, 1993; Bush, 1995), including the phytochrome regulation of gene expression (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994a, 1994b; Millar *et al.*, 1994). Calcium chelators and calcium channel blockers have been used to demonstrate the involvement of calcium in biochemical responses in several plant systems (e.g. Braam, 1992; Bowler *et al.*, 1994b; Kamada and Muto, 1994; Preisig, 1994; Monroy and Dhinsda, 1995; Ohto *et al.*, 1995; Reiss and Beale, 1995). Therefore, the involvement of calcium in the UV-B and UV-A/blue light signalling pathways regulating *CHS* was investigated by examining the effects of various calcium antagonists in the *Arabidopsis* cell culture. Figure 4.1A shows that incubation of *Arabidopsis* cells with increasing concentrations of EGTA attenuated *CHS* transcript accumulation in response to UV-B and UV-A/blue light. However, later data indicate that EGTA may be having a spurious effect in this system (see 4.10 and 4.11).

Addition of the voltage-dependent calcium channel blocker, nifedipine, to the culture medium at 50 μM, inhibited the accumulation of *CHS* transcripts in response to both UV-B and UV-A/blue light treatments. In contrast, incubation of *Arabidopsis* cells with 100 μM verapamil, a second class of voltage-dependent calcium channel blocker, had no inhibitory effect on *CHS* induction by UV-B and UV-A/blue light (Fig. 4.1B). Similarly, *CHS* induction was unaffected when cells were treated with 10 mM lanthanum (Fig. 4.1B), which competes externally with calcium for plasma membrane calcium channels (Tester, 1990). These results suggest that the UV-B and UV-A/blue signalling pathways regulating *CHS* expression involve the activity of a nifedipine-sensitive calcium channel which is insensitive to verapamil or lanthanum. The lack of effect of lanthanum suggests an internal source of calcium, therefore the effect of ruthenium red, which is known to inhibit mitochondrial, endoplasmic reticulum and vacuolar calcium channels (Campbell, 1983; Knight *et al.*, 1992; Allen *et al.*, 1995; Haley, *et al.*, 1995; Monroy and Dhinsda, 1995) was examined. Incubation of *Arabidopsis* cells with 50 μM ruthenium red substantially reduced *CHS* transcript accumulation in response to UV-B and UV-A/blue light treatments (Fig. 4.1B). However, this does not necessarily imply that the calcium requirement is generated from an intracellular source because recent reports indicate that ruthenium red can also act on plasma membrane calcium channels in plants (Marshall *et al.*, 1994). None of the above inhibitors had any effect on the level of *H1* transcripts, indicating that their effects on *CHS* expression were not the result of cell damage causing a general breakdown of transcripts.

4.3 An Increase in Cytosolic Calcium is not Sufficient to Stimulate *CHS*

Since the above results indicated an involvement of cellular calcium in the UV/blue phototransduction pathways regulating *CHS* expression, it was investigated whether the artificial elevation of cytosolic calcium could stimulate *CHS* transcript accumulation.

The calcium ionophore A23187, widely used to study the regulatory role of calcium in biological systems, was added to cells kept in a low fluence rate of white light. The culture medium was also supplemented with 10 mM $CaCl_2$ as studies show that treatment with A23187 alone is not sufficient to elevate cytosolic calcium levels in some plant cells (Suzuki *et al.*, 1995). Braam (1992) reported that *TCH3* gene expression in *Arabidopsis* root cell cultures is stimulated by treatments which elevate cytosolic calcium. *TCH3* transcript levels were therefore measured as a control to show that an increase in cytosolic calcium, sufficient to affect gene expression, had occurred in the *Arabidopsis* cell culture. As shown in Figure 4.2A, the ionophore/calcium treatment caused an increase in *TCH3* transcripts in the *Arabidopsis* cell culture. In contrast to the root cell culture experiments (Braam, 1992), increasing external calcium up to 100 mM in the absence of ionophore failed to induce *TCH3* expression (data not shown). Importantly, no increase in *CHS* transcripts, above that normally present in low white light, was observed in the presence of the ionophore and calcium over a 12 hour period. These results therefore suggest that an increase in cytosolic calcium is insufficient on its own to stimulate *CHS* expression. Support for this conclusion comes from the observation that the calcium channel agonist Bay K8644, used to elevate cytosolic calcium in other plant cell systems (Tretyn *et al.*, 1990; Monroy and Dhindsa, 1995), failed to induce *CHS* transcript accumulation (Fig. 4.2B). In contrast to ionophore/calcium treatment, only a small increase in *TCH3* transcripts was detected in response to Bay K8644 even when the culture medium was supplemented with 10 mM $CaCl_2$ (Fig. 4.2B).

4.4 Calmodulin is Involved in the UV-B but not the UV-A/blue Phototransduction Pathway Regulating *CHS*

Since the inhibitor experiments (Fig. 4.1) indicated a requirement for calcium in the UV-B and UV-A/blue phototransduction pathways, the involvement of calmodulin was investigated. Calmodulin antagonists have been used to demonstrate an involvement of

calmodulin in a number of plant responses (Lam *et al.*, 1989a; Shimazaki *et al.*, 1992; Bowler *et al.*, 1994b; Preiseg and Moreau, 1994; Ohto *et al.*, 1995). Thus the effects of the potent calmodulin antagonist, W-7 (Hidaka *et al.*, 1981), on the UV-B and UV-A/blue light induction of *CHS* were tested in the *Arabidopsis* cell culture. As a control, the effects of the relatively inactive analogue W-5 were also examined. Figure 4.3 shows that *CHS* induction in response to UV-B illumination was dramatically reduced at increasing concentrations of W-7, whereas treatment with W-5, at equivalent concentrations, was without effect. In contrast, W-7 and W-5 had very little effect on the induction of *CHS* transcripts by UV-A/blue light, even at concentrations higher than those shown (up to 200 μM). Since the UV-A/blue light induction of *CHS* was not significantly affected by these calmodulin antagonists, the effect of W-7 on the UV-B response cannot be due to a general inhibition of transcription or acceleration of mRNA turnover. Moreover, the differential effect observed implies that the UV-B and UV-A/blue pathways are, at least in part, distinct. The differential potencies observed between W-7 and W-5 indicate that the target of both these compounds is likely to be calmodulin (Hidaka *et al.*, 1981). Consistent with this is the observation that a second calmodulin antagonist, trifluoperazine, also inhibited *CHS* transcript accumulation in response to UV-B. Similarly, at the same concentration, trifluoperazine abolished the induction of *CHS* by UV-A/blue light. However, in each case, trifluoperazine had no effect on the level of *H1* transcripts, indicating its effects on *CHS* were not due to a general breakdown of transcripts. Since the UV-A/blue phototransduction pathway is insensitive to W-7, it is unlikely that the target of trifluoperazine, in this respect, is calmodulin. As calcium-dependent protein kinases are also sensitive to calmodulin anatagonists (Campbell, 1983; Roberts and Harmon, 1992), it is possible that the UV-A/blue phototransduction pathway regulating *CHS* involves the activity of a trifluoperazine-binding protein other than calmodulin.

4.5 Effects of Ion Channel Blockers on the UV-B and UV-A/blue Light Induction of *CHS* Expression

Blue light has been shown to induce membrane potential changes in a number of plant species (e.g. Assmann *et al.*, 1985; Nishizaki, 1988; Spalding and Cosgrove 1989). Further studies, using ion channel blockers, demonstrate that such potential changes result from ion fluxes across the plasma membrane in response to blue light irradiation (Schwartz *et al.*, 1991, Amodeo *et al.*, 1992; Spalding and Cosgrove, 1992; Nishizaki, 1994; Cho and Spalding, 1996). Therefore, the effects of various ion channel blockers on the UV-B and UV-A/blue light regulation of *CHS* in the *Arabidopsis* cell culture were examined. Figures 4.4A and 4.4B show that incubation of cells with increasing concentrations of the proton-ATPase inhibitor, vanadate, and the potassium channel blocker, tetraethylammonium, had no inhibitory effect on the induction of *CHS* by either UV-B or UV-A/blue light. No inhibition by vanadate was observed even at concentrations higher than those shown (up to 10 mM).

For some plant responses, evidence suggests that blue light inactivates the plasma membrane proton-ATPase (Splading and Cosgrove, 1992; Nishizaki *et al.*, 1994). We therefore investigated whether vanadate could stimulate *CHS* transcript accumulation in *Arabidopsis* cells grown in a low fluence rate of white light. However, no induction of *CHS* in response to either vanadate or tetraethylammonium was observed at the concentrations used in the above experiments (data not shown).

In contrast to the lack of inhibition by vanadate and tetraethylammonium, incubation of cells with increasing concentrations of the anion channel blocker, NPPB, inhibits *CHS* induction in response to both UV-B and UV-A/blue light (Fig. 4.4C). Equivalent concentrations of NPPB have been reported to inhibit anion channel currents in *Vicia* guard cells (Schroeder *et al.*, 1993) and a blue light-activated anion channel in *Arabidopsis* hypocotyl cells (Cho and Spalding, 1996). NPPB had no inhibitory effect at a concentration of 5 μM (data not shown). Levels of *H1* transcripts were unaffected by any of the inhibitor concentrations examined. This indicates that the UV-B and UV-A/blue signalling pathways regulating *CHS* in *Arabidopsis* cells may involve anion

channel activation. However, later data indicate that NPPB may be having a detrimental effect in this cell system (see 4.10 and 4.11).

4.6 Flavin Antagonists Prevent *CHS* transcript accumulation in response to UV-B and UV-A/blue light

Using inhibitors of flavin photoreactions, several studies have demonstrated an involvement of flavins in UV-B and UV-A/blue photoreception (e.g. Short *et al.*, 1992; Warpeha *et al.*, 1992; Khare and Guruprasad, 1993). Indeed, the CRY1 photoreceptor binds a flavin chromophore (Ahmad and Cashmore, 1996). Therefore, the effects of various flavin antagonists on the UV-B and UV-A/blue light induction of *CHS* in the *Arabidopsis* cell culture were examined. As shown in Figure 4.5, incubation of cells with phenylacetic acid, salicylhydroxamic acid and sodium azide, at concentrations shown to be effective in other studies (Short *et al.*, 1992; Warpeha *et al.*, 1992; Khare and Guruprasad, 1993), inhibited the induction of *CHS* in response to UV-B and UV-A/blue light. None of these inhibitors had any effect on the level of *H1* transcripts. These results therefore suggest that flavins may be required for both the UV-B and UV-A/blue light regulation of *CHS* in the *Arabidopsis* cell culture. However, there is evidence that some of these compounds may also affect pterins (Warpeha *et al.*, 1992). Moreover, sodium azide is a well established inhibitor of mitochondrial electron transport. Thus the specificty of these compounds as flavin antagonists is questionable. Indeed, further experiments indicate that the effects of these compounds in *Arabidopsis* cells may result from a general inhibition of transcriptional activation (see 4.10 and 4.11).

4.7 Protein Kinase Inhibitors Prevent the UV-B and UV-A/blue Light Induction of *CHS*

Several studies have demonstrated a role for protein phosphorylation in plant responses by using a variety of protein kinase inhibitors (e.g. Renelt *et al.*, 1993; Bowler *et al.*, 1994b; Kamada and Muto, 1994; MacKintosh *et al.*, 1994; Suzuki *et al.*, 1995). Therefore, the effects of various protein kinase inhibitors on the UV-B and UV-A/blue light regulation of *CHS* in the *Arabidopsis* cell culture system were examined. Figures 4.6A and 4.6B show that incubation of cells with increasing concentrations of the general serine/threonine protein kinase inhibitors, staurosporine and K252a, completely inhibits *CHS* induction in response to both UV-B and UV-A/blue light. Although these compounds are reported to inhibit protein kinase C, we failed to observe any significant increase in *CHS* transcripts, above those normally present in low white light, in response to phorbol myristate acetate (1-10 μM), a potent activator of protein kinase C in animal cells (data not shown). Levels of *H1* transcripts were unaffected at any of the inhibitor concentrations examined. In contrast, the tyrosine/histidine kinase inhibitor, genistein, had no inhibitory effect on the induction of *CHS* by either UV-B or UV-A/blue light (Fig. 4.6C). At equivalent concentrations, genistein completely inhibits the phytochrome transduction pathway regulating *CHS* expression in soybean suspension cultures (Bowler *et al.*, 1994b). This indicates that the UV-B and UV-A/blue signalling pathways regulating *CHS* in *Arabidopsis* cells are different to the phytochrome signalling pathway identified in soybean.

4.8 Protein Phosphatase Inhibitors Prevent the UV-B and UV-A/blue light Induction of *CHS*

Protein phosphatase inhibitors, in addition to protein kinase inhibitors, have been used to demonstrate a role for protein phosphorylation in plant responses (e.g. Renelt *et al.*, 1993; Sheen, 1993; MacKintosh *et al.*, 1994; Takeda *et al.*, 1994a). Therefore, the effect of okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A, on *CHS*

expression in the *Arabidopsis* cell culture was examined. As shown in Figure 4.7A, incubation of cells with 1 μM okadaic acid prevents *CHS* transcript accumulation in response to UV-B and UV-A/blue light treatments. In addition, *CHS* induction by UV-B and UV-A/blue light is abolished after treatment with 100 μM cantharidin, a less potent protein phosphatase inhibitor (Fig. 4.7B). Cantharidin had no inhibitory effect at a concentration of 1 μM (data not shown). Neither inhibitor had any effect on the level of *H1* transcripts. Thus these observations indicate a requirement for phosphatase acivity, in addition to kinase activity, in the UV-B and UV-A/blue photo-transduction pathways regulating *CHS* expression. Ceramide, a sphingolipid-derived second messenger, is reported to stimulate protein phosphatase activity in animal cell systems (Liscovitch and Cantley, 1994). However, addition of ceramide, at concentrations known to be effective in animal cells (Hannun, 1994), failed to induce *CHS* transcript accumulation in *Arabidopsis* cells grown in low intensity white light (data not shown).

4.9 Protein Phosphatase Inhibitors Induce *PAL* Expression

While it was observed that okadaic acid and cantharidin inhibited *CHS* expression in response to UV-B and UV-A/blue light, we also discovered, as shown in Figure 4.8, that these protein phosphatase inhibitors, at equivalent concentrations, could stimulate *PAL* transcript accumulation in cells incubated in a low fluence rate of white light. These results are consistent with the findings of MacKintosh *et al.* (1994) who report that cantharadin, okadaic acid and other protein phosphatase inhibitors stimulate PAL activity and phytoalexin production in soybean cotyledons and suspension cultures. However, MacKintosh *et al.* (1994) did not investigate the effect of protein phosphatase inhibitors on *PAL* transcript levels. The observation that cantharidin and okadaic acid stimulate *PAL* expression in the *Arabidopsis* cell culture demonstrates that the effects of these compounds on *CHS* expression do not result from a general inhibition of transcription or an acceleration of mRNA turnover.

4.10 Effects of Inhibitors on *PAL* Induction by Cantharidin

The above results demonstrate that the effects of okadaic acid and cantharidin on *CHS* expression do not result from a general inhibition of transcription. To satisfy ourselves that the other compounds used in this study were also not generally detrimental to gene expression, their effects on *PAL* induction by cantharidin were examined. Figure 4.9A demonstrates that incubation of cells with nifedipine, ruthenium red, W-7 and trifluoperazine, at concentrations found to inhibit the induction of *CHS* by UV-B and/or UV-A/blue light, have no significant inhibitory effect on this response. However, the addition of 1 μM staurosporine prevented *PAL* induction by cantharidin. This result is consistent with the observation that the protein kinase inhibitor, K252a, at similar concentrations, inhibits the induction of PAL activity in soybean cells (MacKintosh *et al.*, 1994). As shown in Figures 4.9A and 4.9B, incubation of cells with EGTA, NPPB, phenylacetic acid, salicylhydroxamic acid and sodium azide, at concentrations found to inhibit the induction of *CHS* by UV-B and UV-A/blue light, prevented *PAL* transcript accumulation in response to cantharidin. These observations indicate that some of the compounds used in this study may cause a general inhibition of gene expression.

4.11 Effects of Inhibitors on *TCH3* Induction by Ionophore/calcium

To determine whether NPPB, phenylacetic acid, salicylhydroxamic acid and sodium azide cause a general inhibition of gene expression in the *Arabidopsis* cell culture, their effects of *TCH3* induction by A23187/calcium were examined. Figure 4.10 demonstrates that incubation of cells with any of these compounds, at concentrations found to inhibit *CHS* expression, inhibits this response. Thus it seems likely that these compounds, at the concentrations employed, cause a general inhibition of transcription in the *Arabidopsis* cell culture. The observation that staurosporine reduces the induction of *TCH3* transcript accumulation by ionophore/calcium indicates a

requirement for protein kinase activity in this response. However, the possibilty that staurosporine inhibits gene expression in general cannot be excluded.

4.12 Cycloheximide Inhibits the UV-B and UV-A/blue Light Induction of *CHS* Expression

The phytochrome-mediated light induction of *CAB* gene expression has been shown to require protein synthesis since it is inhibited by cycloheximide (Lam *et al.*, 1989b). More recently, Green and Fluhr (1995) have reported that cytoplasmic protein synthesis is required for the UV-B-induced expression of the plant pathogenesis-related protein PR-1 in tobacco leaves. It was therefore investigated whether protein synthesis is required for the UV-B and UV-A/blue light induction of *CHS* expression. As shown in Figure 4.11A, the addition of cycloheximide to the culture medium abolishes the increase in *CHS* transcripts in UV-B and UV-A/blue light. As a control, cycloheximide does not affect the *TCH3* expression in response to A23187/calcium treatment (Fig. 4.11B). Therefore, the inhibition of protein synthesis does not have a general, damaging effect on transcription and transcript accumulation in the *Arabidopsis* cells.

4.13 Activators of the Phytochrome Signalling Pathways Regulating *CHS* are Ineffective in the *Arabidopsis* System

Microinjection experiments with tomato and pharmacological experiments with soybean cells have provided information on the phytochrome signal transduction pathway regulating *CHS* expression (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994a, 1994b). This pathway involves activation of at least one heterotrimeric G protein and cGMP rather than calcium and calmodulin. As shown in Figure 4.12A, no stimulation of *CHS* transcript accumulation was observed following the addition of cell permeable, dibutyryl-cGMP (100 μM) to *Arabidopsis* cells in low intensity white light. Moreover, 100 μM sodium nitroprusside, which dramatically induces *CHS* in soybean cells by

presumably increasing intracellular cGMP concentrations through activation of guanylyl cyclase (Bowler *et al.*, 1994b), does not stimulate *CHS* in the *Arabidopsis* system. However, as a control, a large increase in *CHS* transcripts was observed in response to high intensity white light. Therefore, these results provide further evidence that the UV-B and UV-A/blue light signalling pathways regulating *CHS* in *Arabidopsis* cells are distinct to the phytochrome pathway in tomato and soybean.

Neuhaus *et al.* (1993) reported that both *CHS* and *CAB* promoter activity in tomato are stimulated by cholera toxin, a potent agonist of G proteins (Fig. 1.2). Furthermore, Romero and Lam (1993) demonstrated that incubation of dark-adapted soybean cells with activated cholera toxin induces *CAB* gene expression. It was therefore investigated whether cholera toxin could stimulate *CHS* transcript accumulation in the *Arabidopsis* cell culture. As a control, *CAB* transcript levels were measured to show that G protein activation, sufficient to affect gene expression, had occurred in our cells. As shown in Figure 4.12B, treatment of dark-adpated *Arabidopsis* cells with activated cholera toxin caused a small increase in *CAB* transcripts. However, no increase in *CHS* transcript levels was observed in response to cholera toxin. As a control, treatment of *Arabidopsis* cells with high intensity white light increased both *CHS* and *CAB* transcript levels. These findings suggest that *CHS* induction in *Arabidopsis* cells does not appear to require heterotrimeric G protein activation.

4.14 Identification of a putative G protein α-subunit in *Arabidopsis* membranes

Although the above results suggest that heterotrimeric G protein activation may not be required for *CHS* expression, Warpeha *et al.* (1991) reported that blue light stimulates GTPase activity in membranes isolated from the apical buds of dark-grown pea seedlings. Using antibodies raised to particular animal G protein α-subunits, including α-transducin, they identified a 40 kDa polypeptide which exhibits properties of a blue

light-regulated G protein. It was investigated whether a similar protein could be identified in *Arabidopsis* membranes by using an antibody raised against the C-terminal decapeptide of rat α-transducin provided by Professor Graeme Milligan (Division of Biochemistry and Molecular Biology, University of Glasgow). The extreme C-terminus of each G protein α-subunit appears to be a key domain associated with receptor interaction. This paricular antiserum is specific for α-transducin and other G protein α-subunits (members of the G_i class) which possess an almost identical C-terminal decapeptide sequence (Milligan, 1990). Rat brain membranes (also supplied by Professor Milligan) and pea apical bud membranes were used as a control to demonstrate the antisera could recognise antigens of the correct molecular weight. As shown in Figure 4.13B, the α-transducin antibody identified a single polypetide in rat brain membranes with a molecular weight consistent with that for mammalian G protein α-subunits (40-50 kDa). The α-transducin antibody also recognised a polypepetide in membranes isolated from both dark and light-grown pea apical buds. The molecular weight of this protein is very similar to the 40 kDa α-subunit described by Warpeha *et al.* (1991). Interestingly, a 36 kDa polypeptide was identified in membranes isolated from bolting stems of light-grown *Arabidopsis* plants. However, this polypetide appeared to be absent in membranes isolated from mature, green leaf tissue. The high molecular weight species are likely to be artifacts arising from non-specific binding as a similar band was identified with the molecular weight markers. These preliminary results suggest that a polypeptide, similar to the G protein α-subunit identified in pea, may exist in *Arabidopsis*. Whether the *Arabidopsis* polypeptide is a blue light-activated G protein α-subunit remains to be determined.

4.15 Discussion

To investigate the signal transduction processes involved in the induction of *CHS* gene expression by UV-B and UV-A/blue light we examined the effects of specific agonists and inhibitors in the photomixotrophic *Arabidopsis* cell suspension culture. In addition

to providing information on their components, the findings indicate that the UV-B and UV-A/blue light signal transduction pathways are distinct, at least in part, and that they are different from the phytochrome signal transduction pathway coupled to *CHS* gene expression identified in other species.

4.15.1 The involvement of calcium in the UV-B and UV-A/blue phototransduction pathways regulating *CHS*

Several environmental signals have been shown to alter cytosolic levels of calcium in plant cells, and such changes are likely to be primary events in the triggering of cellular responses (Bush, 1993). However, there are only a few observations in the literature implicating calcium in UV/blue light signal transduction (Murphy, 1988; Shinkle and Jones, 1988; Russ *et al.*, 1991). The experiments described here, with well established calcium antagonists, indicate that calcium is involved in both the UV-B and UV-A/blue phototransduction pathways regulating *CHS* gene expression in the *Arabidopsis* cell culture.

Increases in cytosolic calcium can occur either by an influx of calcium from the extracellular space across the plasma membrane, by an efflux of calcium from intracellular stores, or by a combination of both pathways (Schroeder and Thuleau, 1991; Bush, 1995). Several types of calcium channel have been identified in plant cells which are located not only in the plasma membrane but also in the tonoplast (Johannes *et al.*, 1991; Schroeder and Thuleau, 1991; Allen and Sanders, 1994; Bush, 1995). The voltage-dependent calcium channel blocker, nifedipine, strongly inhibited both the UV-B and UV-A/blue light induction of *CHS* (Fig. 4.1A). Bowler *et al.* (1994b) showed that an equivalent concentration of nifedipine completely inhibited the phytochrome induction of *CAB* in soybean cells and Preisig and Moreau (1994) have used higher concentrations to implicate calcium in the synthesis of phytoalexins in tobacco cell suspensions. Although lanthanum and verapamil are reported efficiently to inhibit calcium channels in higher plants (e.g. Bossen *et al.*, 1988; Schroeder and

Thuleau, 1991; Knight *et al.*, 1992; Messiaen *et al.*, 1993; Preisig and Moreau, 1994; Bush, 1995), these reagents had no effect on the induction of *CHS* in response to UV-B and UV-A/blue light. Similar differential effects of verapamil and nifedipine have been observed in studies of sugar-inducible gene expression in tobacco and sweet potato (Ohto *et al.*, 1995). Therefore, a particular class of calcium channel that is sensitive to nifedipine but insensitive to verapamil appears to be involved in the UV-B and UV-A/blue light-induction of *CHS* expression in the *Arabidopsis* cell culture. Since nifedipine is readily cell permeable, it is impossible to say whether its action is targeted to voltage-dependent calcium channels at the plasma membrane, internal membranes, or both. The lack of effect of lanthanum suggests that an influx of external calcium into the cells may not be required for the UV-B and UV-A/blue responses. This hypothesis is supported by the observation that ruthenium red, which is known to inhibit the release of calcium from intracellular stores (Knight *et al.*, 1992; Allen *et al.*, 1995; Haley *et al.*, 1995; Monroy and Dhindsa, 1995), severely attenuated the UV-B and UV-A/blue responses (Fig. 4.1B). However, recent studies reveal that ruthenium red blocks the activity of a voltage-dependent calcium channel in plasma membrane isolated from *Zea mays* roots (Marshall *et al.*, 1994). It is therefore unclear whether ruthenium red and nifedipine exert their effects on the same or different calcium channels in our system. More information is needed to determine whether the calcium requirement is extracellular, intracellular or both. The cantharidin stimulation of *PAL* expression was not affected by any of these compounds, demonstrating that their effects on *CHS* expression did not result from a general inhibition of transcriptional activation or an acceleration of mRNA turnover (Fig. 4.9A). Moreover, none of these compounds caused browning or bleaching of the cells. Some reagents, not described here, did have such effects and were obviously toxic to the cells. Our results therefore indicate that there is an involvement of calcium in both the UV-B and UV-A/blue phototransduction pathways controlling *CHS* in the *Arabidopsis* cell culture and suggest that the most likely source of the calcium is from internal stores. In contrast, the inhibitory effects of EGTA (Fig. 4.1A) suggest that an influx of extracellular

calcium is required for the both the UV-B and UV-A/blue light induction of *CHS*. Braam (1992) has used higher concentrations of EGTA to demonstrate a role for extracellular calcium in the regulation of *TCH* gene expression in *Arabidopsis* root cell cultures. However, EGTA also inhibited the cantharidin induction of *PAL* expression (Fig. 4.9A). These findings make it difficult to conclude whether the effects of EGTA on *CHS* transcript accumulation result from the chelation of extracellular calcium or from a general inhibition of gene expression.

The artificial elevation of cytosolic calcium, by treating cells with the calcium ionophore A23187 or the calcium channel agonist Bay K8644, was insufficient to increase *CHS* transcript levels in low white light (Fig. 4.2). The increase in *TCH3* transcripts in each experiment provides good evidence that these treatments did elevate cytosolic calcium. Similarly, although calcium has been implicated in phytochrome signal transduction, treatment of soybean cells with ionomycin only weakly induced *CAB* gene expression (Lam *et al.*, 1989a). It is possible that UV-B and UV-A/blue light trigger an increase in calcium that is either not cytosolic (for example, nuclear) or occurs in a particular subcellular domain inaccessible to the ionophore. For example, Knight *et al.* (1996) have measured a cold-induced increase in calcium in the microdomain associated with the vacuolar membrane by using *Arabidopsis* containing aequorin targeted to the cytosolic face of the vacuole. Additional experiments are required to determine whether UV/blue light induces an increase in calcium in a particular cellular location. A further possible interpretation of the A23187 and Bay K8644 experiments is that an increase in cytosolic calcium, though necessary, can only stimulate *CHS* expression by acting in conjuction with some other UV/blue light-induced signalling process. Indeed, this may be the case regardless of the location of the calcium pool. The complexity of the transduction processes, discussed in more detail below, supports this possiblity.

4.15.2 The UV-B and UV-A/blue light signal transduction pathways regulating *CHS* differ in the involvement of calmodulin

Calcium can regulate the activities of target proteins directly or via calcium binding proteins, such as calmodulin. The inhibitory effect of the well characterised calmodulin antagonist W-7 on the UV-B-induction of *CHS* indicates that calmodulin activation is required (Fig. 4.3). Consistent with this conclusion is the observation that the less effective analogue, W-5, has no effect on *CHS* induction by UV-B. In contrast, the UV-A/blue light induction of *CHS* was not affected by either inhibitor, demonstrating that the effects of UV-A/blue light on *CHS* are not mediated by calmodulin in this system. The differential effect of W-7 also shows that the calmodulin antagonist does not generally inhibit transcriptional activation. W-7 has been used widely to demonstrate calmodulin involvement in other plant responses (Lam *et al.*, 1989a; Shimizaki *et al.*, 1992; Shimazaki *et al.*, 1993; Preisig and Moreau, 1994; Ohto *et al.*, 1995). However, it should be noted that, in the present experiments, inhibition was observed at the same concentration used in animal cells (10-30 μM). This is important, because at higher concentrations calmodulin antagonists can inhibit other target proteins such as calcium-dependent protein kinases (Campbell, 1983; Roberts and Harmon, 1992). A second calmodulin antagonist, trifluoperazine, strongly inhibited both the UV-B and UV-A/blue light induction of *CHS*. Trifluoperazine had no effect on the cantharidin stimulation of *PAL* expression (Fig. 4.9A), demonstrating that its effects on *CHS* did not result from a general inhibition of transcription or mRNA turnover. The inhibitory effect of trifluoperazine on the induction of *CHS* by UV-B provides further support that calmodulin is involved. However, the differential effect of W-7 and trifluoperazine on the UV-A/blue light induction of *CHS* is not consistent with a role for calmodulin in this response. The UV-A/blue pathway is likely to involve a calcium-dependent or calcium-binding component other than calmodulin which is unaffected by W-7. Taken together, these results indicate that the UV-B and UV-A/blue light signal transduction pathways are, at least in part, distinct. This is consistent with previous

findings, based on experiments with the *hy4* cell culture, that these pathways are initiated by separate photoreceptor systems (Fig 3.9).

4.15.3 The UV/blue phototransduction pathways do not appear to involve H^+ or K^+ fluxes

Blue light triggers stomatal opening by inducing the swelling of stomatal guard cells. Evidence suggests that this process involves redox reactions and/or a rapid activation of the plasma membrane proton-ATPase which results in the influx of K^+ (Kaufman, 1993; Jenkins *et al.*, 1995). Several studies have indicated a role for H^+ and K^+ fluxes in plant responses by using specific inhibitors of ion pumps and channels (Schwartz *et al.*, 1991; Spalding and Cosgrove, 1992; Nishizaki *et al.*, 1994; Elzenga *et al.*, 1995). For example, Yoshioka *et al.* (1992) reported that the plasma membrane proton-ATPase inhibitor, vanadate, delays *CHS* transcript accumulation in pea epicotyls in response to fungal elicitor. At equivalent concentrations, vanadate does not inhibit *CHS* induction by UV-B and UV-A/blue light (Fig. 4.4A). Similarly, the K^+ channel blocker, tetraethylammonium (TEA), at concentrations used in other studies (Spalding and Cosgrove, 1992; Elzenga *et al.*, 1995), has no inhibitory effect on *CHS* expression (Fig. 4.4B). Taken together, these results suggest that the UV/blue phototransduction pathways regulating *CHS* in the *Arabidopsis* cell culture do not involve the activation of K^+ channels or the plasma membrane proton-ATPase. Nevertheless, it is possible that the lack of effect of vanadate or TEA in *Arabidopsis* cells is due to a lack of uptake. Vanadate uptake is reported to be impaired in solutions of high ionic strength (Schwartz *et al.*, 1991). Thus, for these experiments, there is no positive control to demonstrate that vanadate and TEA are effective in the *Arabidopsis* cell system.

4.15.4 Protein phosphorylation appears to be involved in the regulation of *CHS* and *PAL*

Several studies have implicated protein phosphorylation in UV/blue light signal transduction (Kaufman, 1993; Short and Briggs, 1994; Jenkins *et al.*, 1995). Probably the best characterised example is the blue light regulated protein kinase activity identifed by Briggs and co-workers (Short and Briggs, 1994; Liscum and Briggs, 1995) which is an early event in the blue light phototransduction pathway mediating phototropism (Short and Briggs, 1994; Liscum and Briggs, 1995; Jenkins *et al.*, 1995). Further studies, using inhibitors of animal protein kinases, provide evidence for the involvement of protein phosphorylation in the blue light regulation of stomatal opening (Shimazaki *et al.*, 1992; Shimazaki *et al.*, 1993). In our experiments, the inhibitory effects of staurosporine and K252a indicate a requirement for serine/threonine protein phosphorylation in the UV-B and UV-A/blue light induction of *CHS* (Fig. 4.6A and B).

The inhibitory effects of okadaic acid and the less potent protein phosphatase (PP) inhibitor, cantharidin (MacKintosh *et al.*, 1994), suggest that *CHS* induction requires a protein de-phosphorylation event(s) in addition to protein kinase activity (Fig. 4.6). Cantharidin is reported to be an inhibitor of PP2A (Li and Casida, 1992) whereas okadaic acid has been shown, in cell-free extracts, to inhibit PP2A (1 nM) and PP1 at higher concentrations (1 μM) (Cohen *et al.*, 1990). Nevertheless, the present data do not allow us to conclude whether PP1 and/or PP2A is involved in the UV-B and UV-A/blue light-induction of *CHS* because precise concentration-dependent effects of okadaic acid are more difficult to interpret in intact cells. Interestingly, Sheen (1993) has shown that the light-regulated expression of two photosynthetic genes requires a protein phosphatase activity which is likely to be PP1. The observation that these phosphatase inhibitors can stimulate *PAL* expression in the *Arabidopsis* cell culture demonstrates that their effects on *CHS* expression are not due to a general inhibition of transcriptional activation (Fig. 4.8). These data extend the observations of MacKintosh et al (1994) who reported the stimulation of PAL activity by protein phosphatase

inhibitors in soybean. The fact that *CHS* induction is prevented by both protein kinase and phosphatase inhibitors indicates the complexity of the transduction processes. In a simple system, these inhibitors would be expected to have opposing effects, as seen with *PAL* expression. It therefore appears that the regulation of *CHS* may involve separate signalling processes in which these inhibitors have differential effects. This is consistent with the hypothesis developed in relation to the A23187 and Bay K8644 experiments, that both UV-B and UV-A/blue light initiate more than one transduction process.

4.15.5 Protein synthesis is required for the induction of *CHS* by UV-B and UV-A/blue light

The results (Fig. 4.11) also demonstrate that the induction of *CHS* by UV-B and UV-A/blue light in *Arabidopsis* cells requires cytoplasmic protein synthesis. It is most likely that one or more components, such as a transcription factor, is synthesised that is essential for the stimulation of expression. Indeed, expression of CPRF1, a transcription factor that binds to the parsley *CHS* promoter, is rapidly light-induced in parsley cells and plants (Weisshaar *et al.*, 1991a; Feldbrügge *et al.*, 1994). However, not all blue light signal transduction coupled to gene expression requires protein synthesis, since Marrs and Kaufman (1991) observed that cycloheximide had no effect on the blue light regulation of two genes in pea. This provides some evidence that different UV-A/blue phototransduction pathways function to regulate the expression of specific genes. In addition, the observation that the UV-B and UV-A/blue light induction of *CHS* requires the synthesis of an essential component raises the question of whether the signalling events described here are concerned directly with *CHS* transcription or, at least in part, with the expression of genes encoding some other component(s).

4.15.6 The UV-B and UV-A/blue phototransduction pathways appear distinct to the phytochrome pathway regulating *CHS* in other species

Several components of the phytochrome signalling pathway regulating *CHS* expression in tomato and soybean have been identified (Neuhaus, 1993; Bowler *et al.*, 1994a, 1994b). This pathway requires heterotrimeric G protein activation, cGMP and is inhibited by genistein, an inhibitor of tyrosine/histidine kinases in animal cells. The results presented here indicate that the UV-B and UV-A/blue light signal transduction pathways regulating *CHS* in *Arabidopsis* cells are distinct to the phytochrome pathway in tomato and soybean. Firstly, the UV-B and UV-A/blue pathways both involve calcium, in contrast to the phytochrome pathway regulating *CHS*. Secondly, no stimulation of *CHS* transcript accumulation was observed following the addition of dibutyryl-cGMP to *Arabidopsis* cells in low intensity white light (Fig. 4.12A). Moreover, sodium nitroprusside, an activator of guanylyl cyclase, does not induce *CHS* in the *Arabidopsis* system. Bowler *et al.* (1994a, 1994b) have shown that equivalent concentrations of dibutyryl-cGMP and sodium nitroprusside dramatically increase *CAB* transcript levels in soybean cells. Finally, genistein does not inhibit *CHS* expression at a concentration which abolishes the phytochrome induction of *CHS* in soybean cells (Fig. 4.6C) (Bowler *et al.*, 1994b). Given that genistein is effective in soybean cells, it seems very unlikely that its lack of effect in *Arabidopsis* cells is due to a lack of uptake. Taken together, these observations indicate that the UV/blue phototransduction pathways controlling *CHS* expression in *Arabidopsis* cells are different to the phytochrome signalling pathway identified in soybean. Consistent with this, the UV-B and UV-A/blue light induction of *CHS* transcript accumulation in *Arabidopsis* cells does not show the rapid transient increase characteristic of the phytochrome induction of *CHS* in soybean cells (Fig. 3.5) (Bowler *et al.*, 1994a, 1994b). Furthermore, no induction of *CHS* was observed in dark-adapted *Arabidopsis* cells treated with cholera toxin (Fig. 4.7B). The small increase in *CAB* transcripts in the same experiment suggests that G protein activation had occurred in these cells.

Romero and Lam (1993) have shown that a similar concentration of cholera toxin induces *CAB* gene expression in soybean cells. More recently, Neuhaus *et al.* (1993) demonstrated that microinjection of cholera toxin into hypocotyl cells of tomato stimulates both *CHS* and *CAB* promoter activity, presumably through the activation of one or more heterotrimeric G proteins. These experiments therefore suggest that the UV/blue phototransduction pathways regulating *CHS* in *Arabidopsis* cells do not involve heterotrimeric G protein activation.

Interestingly, a putative 36 kDa G protein α-subunit was identified in *Arabidopsis* membranes using an antibody raised to the C-terminal decapeptide of α-transducin (Fig. 4.8B). This polypeptide is detectable in membranes isolated from bolting stem tissue but undetectable in membranes prepared from mature green leaves. Antibodies raised against mammalian G protein α-subunits have previously been used to identify corresponding polypeptides in several plant species, including *Arabidopsis* (Blum *et al.*, 1988; Jacobs *et al.*, Terryn *et al.*, 1993; Kaufman, 1994). Using an antibody raised against α-transducin, Warpeha *et al.* (1991) identified a 40 kDa polypeptide in plasma membranes isolated from dark-grown pea apices. This polypeptide appears to be an α-subunit of a blue light-activated heterotrimeric G protein whose threshold fluence for blue light excitation resembles that for blue light-induced *CAB* gene expression in pea (Kaufman, 1993). However, in preliminary studies with membranes isolated from either dark-grown pea apices or dark-adapted *Arabidopsis* bolting stems, no significant increase in GTPase activity in response to blue light was detected (data not shown). Carbachol, an agonist of G protein coupled muscarinic acetylcholine receptors, elicited a large increase in GTPase activity when added to rat brain membranes, confirming the efficiency of the assay. Consistent with these findings, Warpeha and co-workers have found it difficult to reproduce the blue light-stimulated increase in G protein activity in dark-grown pea apical bud membranes (K.H.F. Warpeha, personal communication). A simpler method would be to look for a blue light stimulation of binding of the non-hydrolysable analogue GTP[γ^{35}S]. This would provide some indication of whether the *Arabiopsis* polypeptide identified here is

in fact an α-subunit of a blue light-regulated G protein. The *Arabidopsis* cell suspension culture may be a useful tool in characterising this protein in more detail.

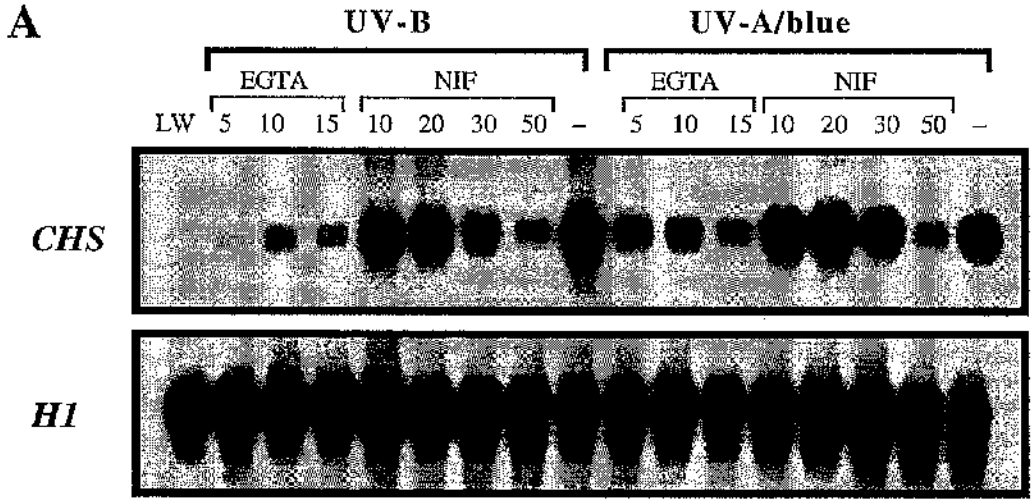
4.15.7 Several compounds appear to cause a general inhibition of gene expression

Flavins are likely candidates for the chromophores of UV-B and UV-A/blue photoreceptors (Briggs and Iino, 1983; Galland and Senger, 1988a). Support for this hypothesis has come from experiments with compounds known to interact with flavin molecules (Short *et al.*, 1992; Warpeha *et al.*, 1992; Khare and Guruprasad, 1993). More recently, direct evidence for the involvement of flavins in UV/blue photoreception has been obtained from the identification and characterisation of the CRY1 photoreceptor (Ahmad and Cashmore, 1996). The flavin antagonists, phenylacetic acid, salicylhyroxamic acid and sodium azide, strongly inhibited both the UV-B and UV-A/blue light induction of *CHS* (Fig. 4.5). However, at equivalent concentrations, these compounds also affected the cantharidin stimulation of *PAL* expression (Fig. 4.9B) and the ionophore/calcium induction of *TCH3* (Fig. 4.10). Similar non-specifc effects were observed with the anion channel blocker, NPPB, when used at 25 μM. These observations therefore indicate that phenylacetic acid, salicylhydroxamic acid, sodium azide and NPPB, at the concentrations employed, may have a general inhibitory effect on gene expression in the *Arabidopsis* cell culture system. Moreover, in each experiment, these compounds had no effect on the *H1* transcript levels. This demonstrates that consitutively expressed transcripts, such as *H1*, do not provide ideal controls for assessing whether particular reagents are generally detrimental to gene expression. Caution should be exercised when interpreting such data.

Figure 4.1. Effects of calcium antagonists on the UV-B and UV-A/blue light induction of *CHS* expression

(A) Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with increasing concentrations of EGTA (mM) or nifedipine (NIF) (μM) prior to illumination with UV-B (3 μmol m^{-2} s^{-1}) or UV-A/blue light (80 μmol m^{-2} s^{-1}). Cells were harvested for RNA extraction after 6 h exposure to UV-B or UV-A/blue light. Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

(B) Cells grown as in (A) were incubated for 1h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with 100 μM (VER), 10 mM lanthanum chloride (LA) or 50 μM ruthenium red (RR) prior to illuminations as in (A). Transcripts were analysed as in (A).


A
UV-B
UV-A/blue
EGTA
NIF
EGTA
NIF
LW 5 10 15 10 20 30 50 – 5 10 15 10 20 30 50 –
CHS
H1

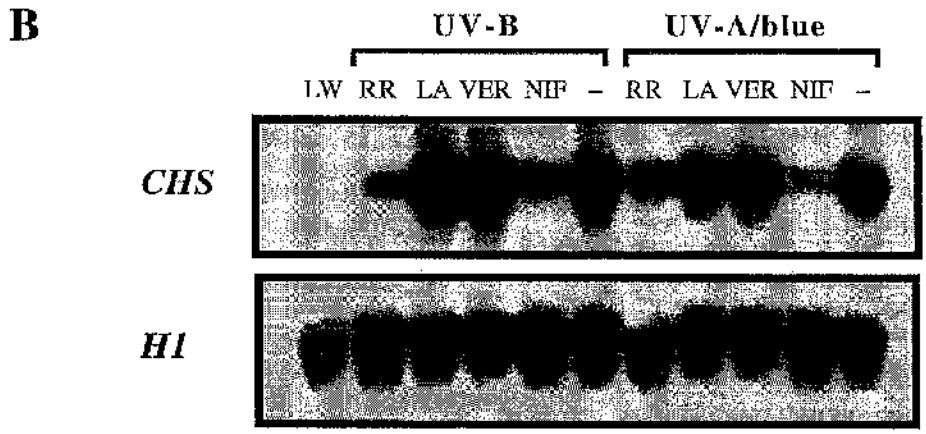
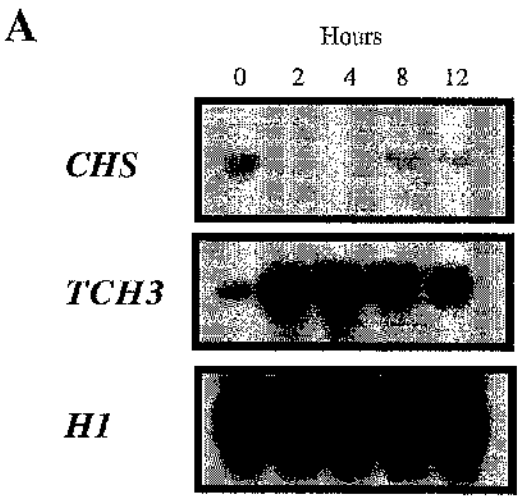

B
UV-B
UV-A/blue
LW RR LA VER NIF – RR LA VER NIF –
CHS
H1

Figure 4.2. Elevating cytosolic calcium increases *TCH3* but not *CHS* transcripts levels

(**A**) Cells grown in 20 μmol m^{-2} s^{-1} white light were treated with 10 μM A23187 and 10 mM $CaCl_2$ and incubated in 20 μmol m^{-2} s^{-1} white light for the times indicated. Equal amounts of RNA (20 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis TCH3* probe then a *Phaseolus vulgaris H1* probe.

(**B**) Cells grown in 20 μmol m^{-2} s^{-1} white light (LW) were treated either without (–) or with 1 or 10 μM Bay K8644 and incubated in 20 μmol m^{-2} s^{-1} white light for 6 h. Transcripts were analysed as in (A).


A
Hours
0 2 4 8 12
CHS
TCH3
H1

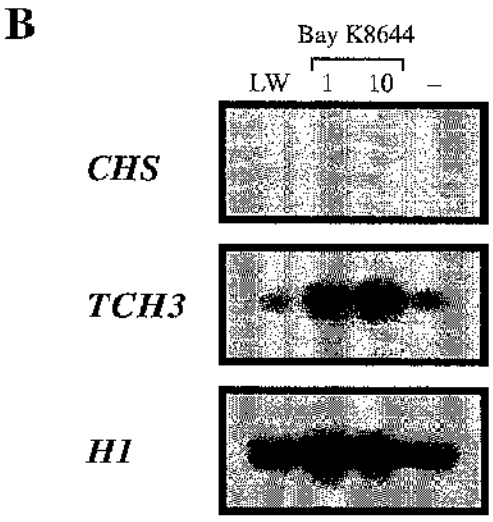

B
Bay K8644
LW 1 10 –
CHS
TCH3
H1

Figure 4.3. Effects of calmodulin antagonists on the UV-B and UV-A/blue light induction of *CHS* expression

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with increasing concentrations (μM) of W-7, W-5 or 25 μM trifluoperazine (TFP) prior to illumination for 6 h with UV-B (3 μmol m^{-2} s^{-1}) or UV-A/blue light (80 μmol m^{-2} s^{-1}). Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

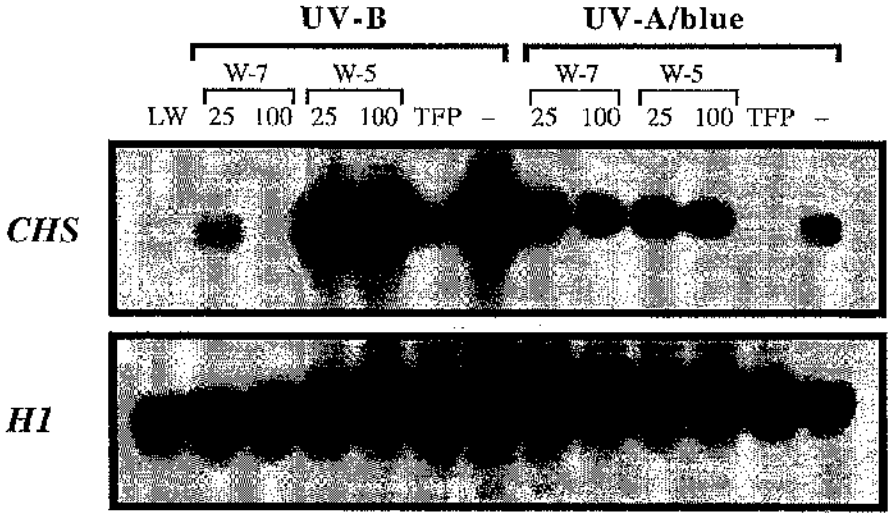
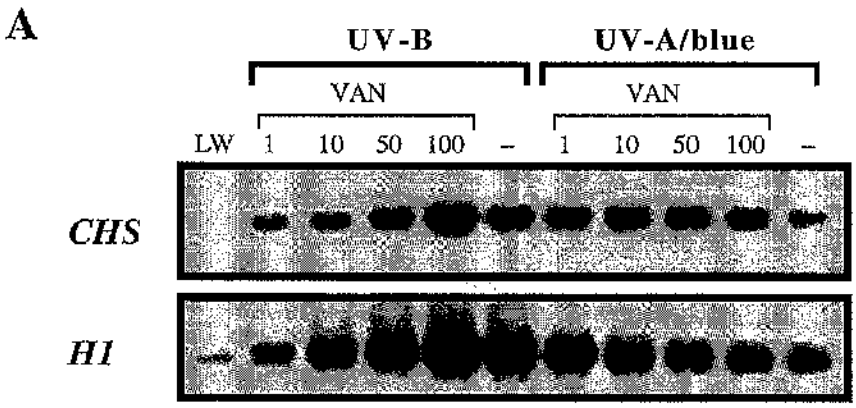

UV-B
UV-A/blue
W-7
W-5
W-7
W-5
LW 25 100 25 100 TFP –
25 100 25 100 TFP –
CHS
H1

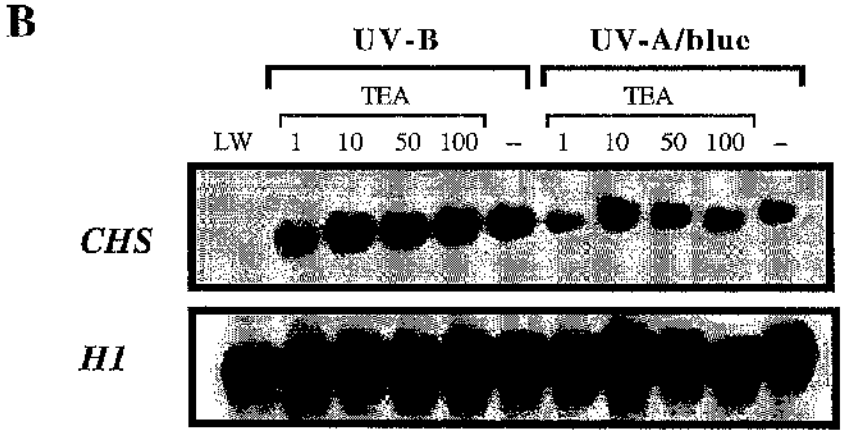
Figure 4.4. Effects of ion channel blockers on the UV-B and UV-A/blue light regulation of *CHS* expression

(A) Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with increasing concentrations (μM) of sodium orthovanadate (VAN) prior to illumination with UV-B (3 μmol m^{-2} s^{-1}) or UV-A/blue light (80 μmol m^{-2} s^{-1}). Cells were harvested for RNA extraction after 6 h exposure to UV-B or UV-A/blue light. Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

(B) Cells grown as in (A) were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with increasing concentrations (mM) of tetraethylammonium chloride (TEA) prior to illumination as in (A). Transcripts were analysed as in (A).

(C) Cells grown as in (A) were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with 25 or 50 μM 5-nitro-2,3-phenylpropylaminobenzoic acid (NPPB) prior to illumination as in (A). Transcripts were analysed as in (A).


A
UV-B
UV-A/blue
VAN
VAN
LW 1 10 50 100 – 1 10 50 100 –
CHS
H1


B
UV-B
UV-A/blue
TEA
TEA
LW 1 10 50 100 – 1 10 50 100 –
CHS
H1

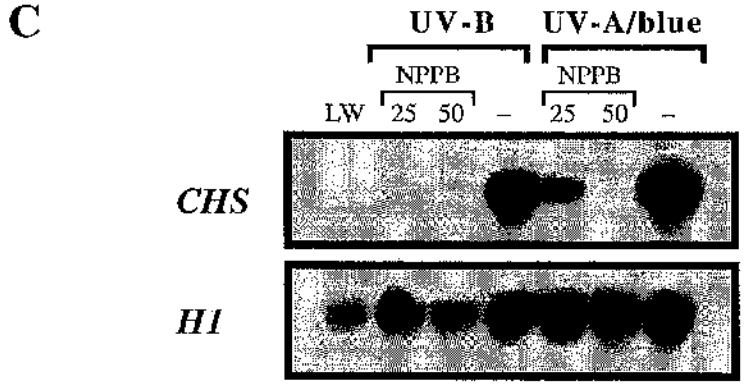

C
UV-B
UV-A/blue
NPPB
NPPB
LW 25 50 – 25 50 –
CHS
H1

Figure 4.5. Effects of flavin antagonists on the UV-B and UV-A/blue light regulation of *CHS* expression

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with increasing concentrations (mM) of phenylacetic acid (PAA), salicylhyroxamic acid (SHAM) or 50 or 100 μM sodium azide (NaN_3) prior to illumination with UV-B (3 μmol m^{-2} s^{-1}) or UV-A/blue light (80 μmol m^{-2} s^{-1}). Cells were harvested for RNA extraction after 6 h exposure to UV-B or UV-A/blue light. Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

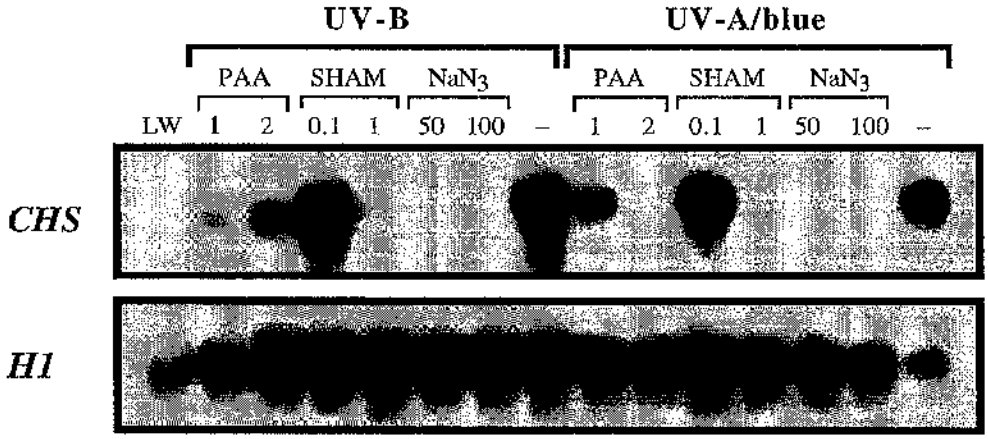

UV-B
UV-A/blue
PAA
SHAM
NaN3
PAA
SHAM
NaN3
LW 1 2 0.1 1 50 100 – 1 2 0.1 1 50 100 –
CHS
H1

Figure 4.6. Effects of protein kinase inhibitors on the UV-B and UV-A/blue light induction of *CHS* transcripts

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with **(A)** 1 or 10 μM staurosporine (ST), **(B)** 1 or 5 μM K252a or **(C)** 75 μM genistein (GEN) prior to illumination for 6 h with UV-B (3 μmol m^{-2} s^{-1}) or UV-A/blue light (80 μmol m^{-2} s^{-1}). Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

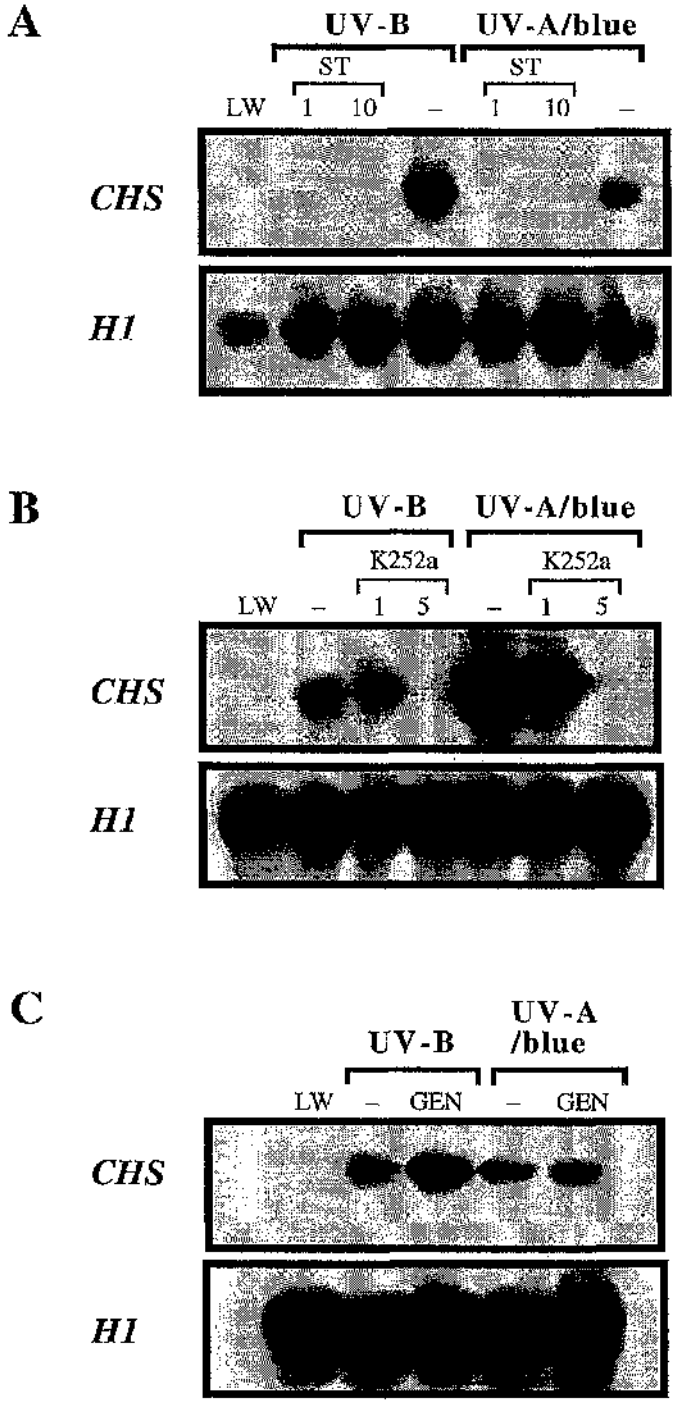

A
UV-B
UV-A/blue
ST
ST
LW
1
10
–
1
10
–
CHS
H1
B
UV-B
UV-A/blue
K252a
K252a
LW
–
1
5
–
1
5
CHS
H1
C
UV-B
UV-A
/blue
LW
–
GEN
–
GEN
CHS
H1

Figure 4.7. Effects of protein phosphatase inhibitors on the UV-B and UV-A/blue light induction of *CHS* expression

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with **(A)** increasing concentrations (nM) of okadaic acid (OKA) or **(B)** 100 μM cantharidin (CAN) prior to illumination for 6 h with UV-B (3 μmol m^{-2} s^{-1}) or UV-A/blue light (80 μmol m^{-2} s^{-1}). Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

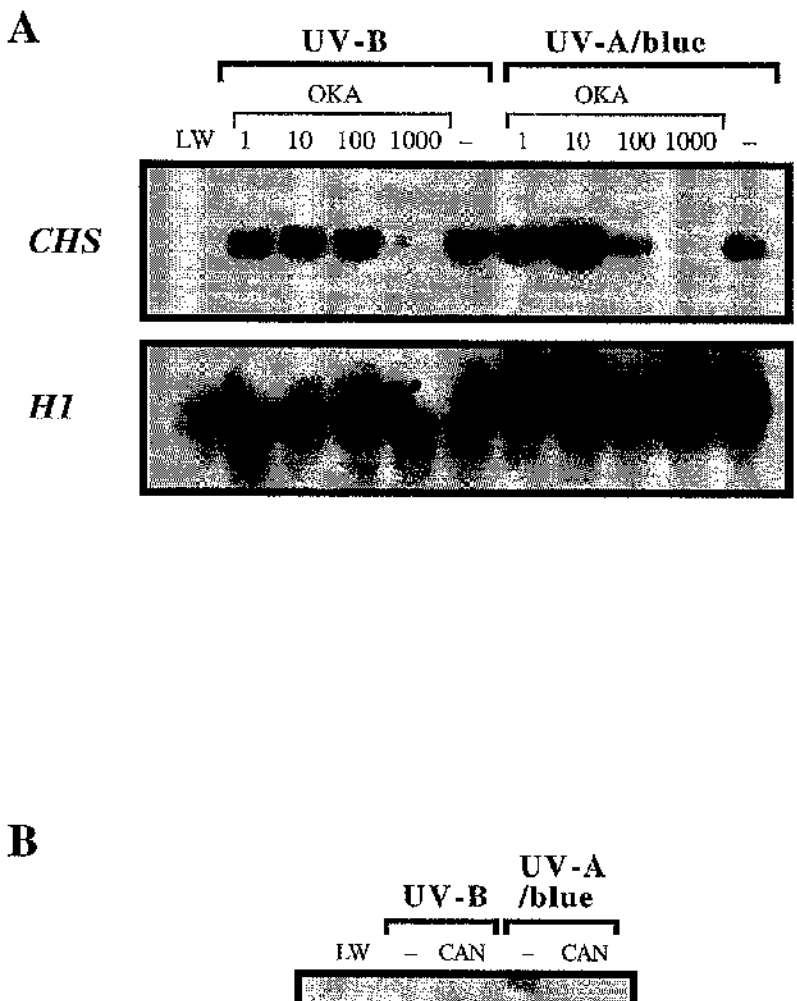

A
UV-B
UV-A/blue
OKA
OKA
LW 1 10 100 1000 – 1 10 100 1000 –
CHS
H1
B
UV-B
UV-A /blue
LW – CAN – CAN
CHS
H1

Figure 4.8. Protein phosphatase inhibitors stimulate *PAL* transcript accumulation

(A) Cells grown in 20 µmol m^{-2} s^{-1} white light (LW) were incubated for 6 h in 20 µmol m^{-2} s^{-1} white light either without (–) or with 1 µM okadaic acid (OKA). Equal amounts of RNA (20 µg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. The membrane was then stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

(B) Cells grown as in (A) were incubated for 6 h in 20 µmol m^{-2} s^{-1} white light either without (–) or with 100 or 200 µM cantharidin (CAN). Transcripts were analysed as in (A).

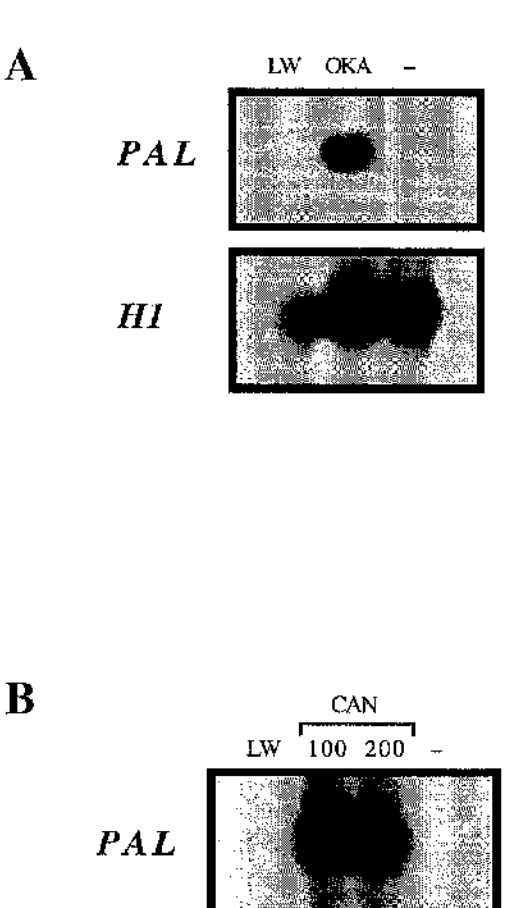
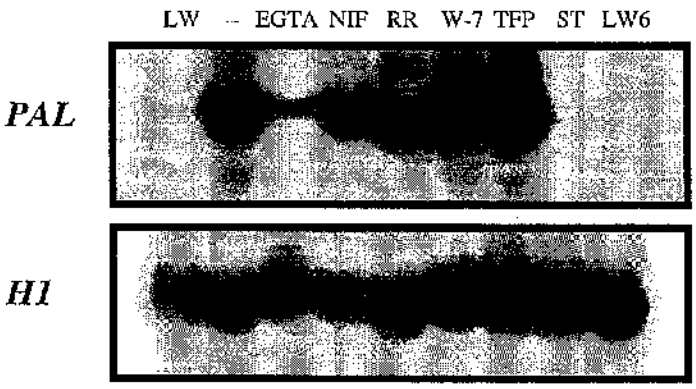

A
LW OKA -
PAL
H1
B
CAN
LW 100 200 -
PAL
H1

Figure 4.9. Effects of various signalling antagonists on the cantharidin induction of *PAL* gene expression

Cells grown in 20 μmol m^{-2} s^{-1} white light (LW) were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light either without (–) or with **(A)** 10 mM EGTA, 50 μM nifedipine (NIF), 50 μM ruthenium red (RR), 25 μM W-7, 25 μM trifluoperazine (TFP), **(B)** 25 μM NPPB, 1mM phenylacetic acid (PAA), 1 mM salicylhydroxamic acid (SHAM), or 50 μM sodium azide (NaN_3) prior to the addition of 200 μM cantharidin. Cells were harvested after 6 h and total RNA isolated. LW6 represents cells incubated in low white light for a further 6 h without a cantharidin or ionophore/calcium treatment. Equal amounts of RNA (20 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. The membrane was then stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

A


LW – EGTA NIF RR W-7 TFP ST LW6
PAL
H1

B

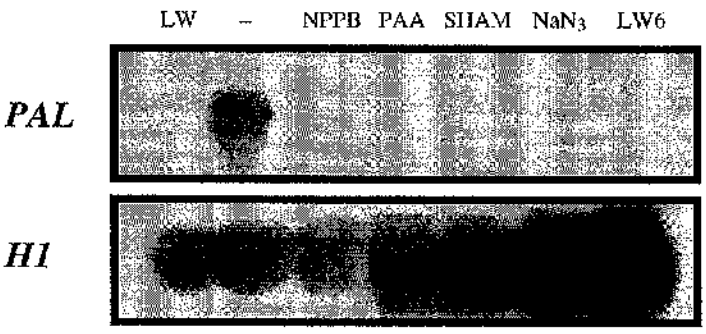

LW – NPPB PAA SHAM NaN3 LW6
PAL
H1

Figure 4.10. Effects of various signalling antagonists on the ionophore/calcium induction of *TCH3* expression

Cells grown in 20 μmol m^{-2} s^{-1} white light (LW) were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light either without (–) or with 1 μM staurosporine (ST), 1mM phenylacetic acid (PAA), 1 mM salicylhydroxamic acid (SHAM), 50 μM sodium azide (NaN_3) or 25 μM NPPB prior to the addition of 10 μM A23187 and 10 mM $CaCl_2$. Cells were harvested after 6 h and total RNA isolated. Equal amounts of RNA (20 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. The membrane was then stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

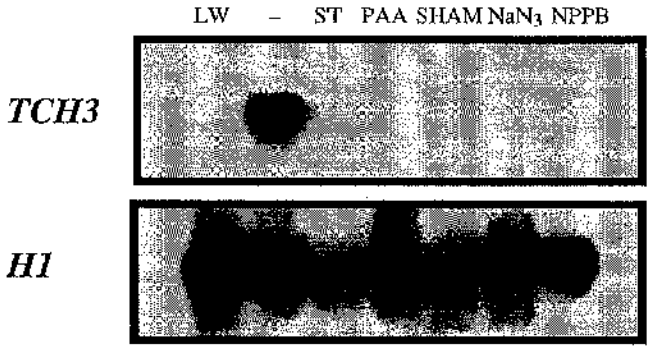
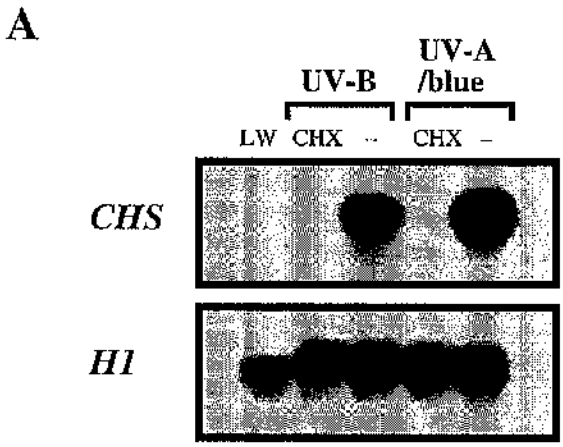

LW
–
ST
PAA
SHAM
NaN3
NPPB
TCH3
H1

Figure 4.11. The effects of cycloheximide on *CHS* and *TCH3* gene expression

(A) Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with 20 μM cycloheximide (CHX) prior to illumination for 6 h with UV-B (3 μmol m^{-2} s^{-1}) or UV-A/blue light (80 μmol m^{-2} s^{-1}). Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

(B) Cells were grown and incubated either without (–) or with 20 μM cycloheximide (CHX) as in (A). 10 μM A23187 and 10 mM $CaCl_2$ were then added and cells harvested after 6 h. A northern blot of total RNA (20 μg per lane) was hybridised to the *Arabidopsis TCH3* probe and rehybridised to the *Phaseolus vulgaris H1* probe.


A
UV-B
UV-A
/blue
LW CHX –
CHX –
CHS
H1

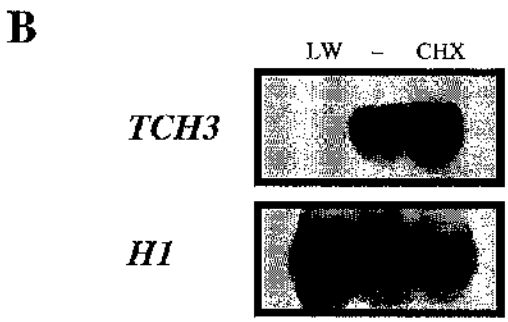

B
LW – CHX
TCH3
H1

Figure 4.12. Mediators of phytochrome signalling do not stimulate *CHS* transcript accumulation

(A) Cells grown in 20 μmol m^{-2} s^{-1} white light (LW) were incubated without (–) or with 100 μM dibutyryl-cGMP or 100 μM SNP and incubated in 20 μmol m^{-2} s^{-1} white light for 6 h. As a positive control, cells were illuminated for 6 h with 120 μmol m^{-2} s^{-1} white light (HW). Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was hybridised to an *Arabidopsis CHS* probe, washed and subjected to autoradiography. The membrane then stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

(B) On the third day after subculture, cell culture flasks were wrapped in 2 layers of aluminium foil and returned to the shaker for a further 2 days. Cells grown in the dark (D) were incubated without (–) or with 0.5 or 1 μg ml^{-1} activated cholera toxin (CT) and incubated in the dark for 6 h. As a positive control, cells were illuminated for 6 h with 120 μmol m^{-2} s^{-1} white light (HW). Total RNA was isolated and separated as in (A). A northern blot was hybridised to an *Arabidopsis CHS* probe, washed and subjected to autoradiography. The membrane was then stripped of radioactivity and reprobed with an *Arabidopsis CAB* probe and the *Phaseolus vulgaris H1* probe.

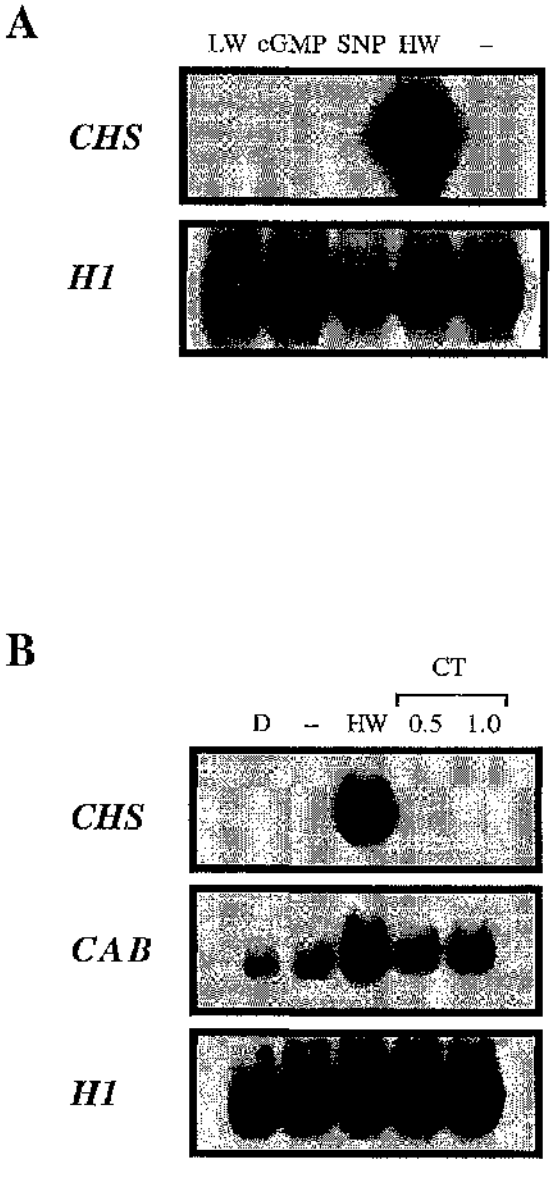
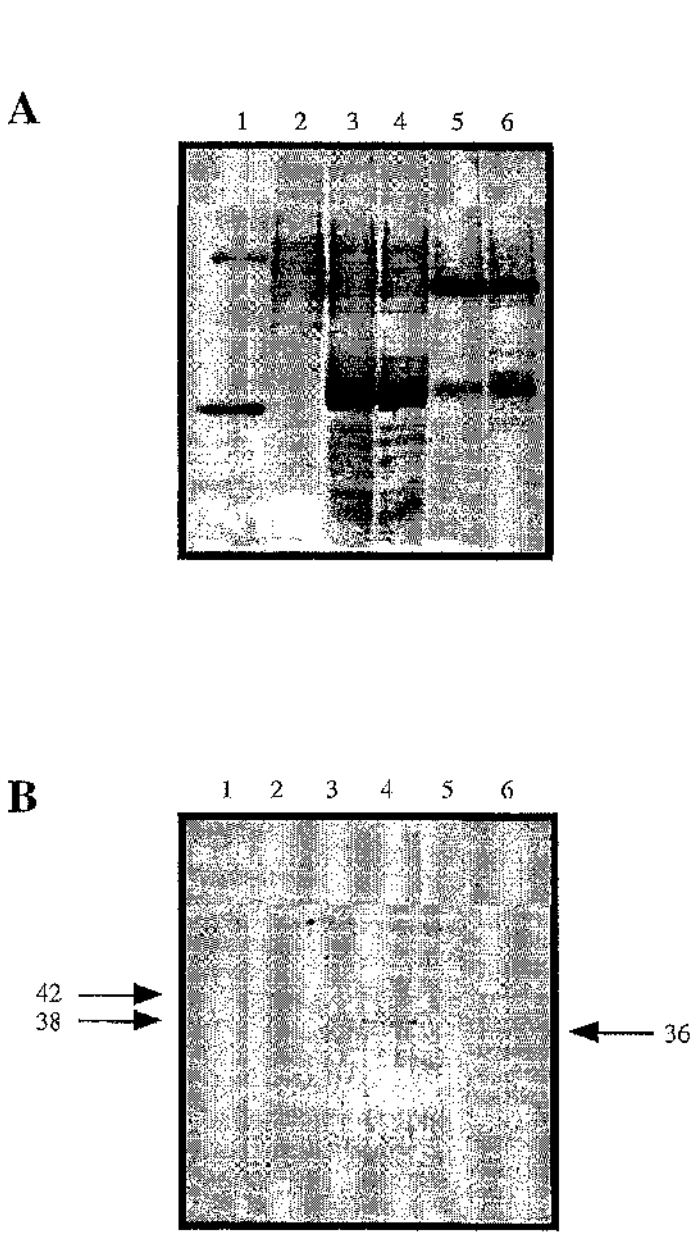

A
LW cGMP SNP HW –
CHS
H1
B
CT
D – HW 0.5 1.0
CHS
CAB
H1

Figure 4.13. Immunoblot analysis of *Arabidopsis* and pea membrane proteins using a polyclonal antibody raised against the C-terminal decapeptide of α-transducin

(A) Membrane proteins were isolated from rat brain membranes (lane 2), 7 day old dark-grown pea apical buds (lane 3), 7 day old light-grown pea apices (lane 4), 21 day old light-grown *Arabidopsis* leaf tissue (lane 5) and 21 day old light-grown *Arabidopsis* flowering stem tissue (lane 6). Proteins were resolved by electrophoresis on a 12.5% polyacrylamide SDS gel before electrotransfer to a nitrocellulose filter. The filter was stained with amidoblack to visualise the polypeptide samples. The molecular weight markers used (lane 1) are: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

(B) A duplicate filter of that shown in (A) was probed with a polyclonal antibody raised to the C-terminal decapeptide of rat α-transducin. Lanes 1-6 correspond to those described in (A). Molecular masses of the peptides identified (kDa) are indicated.


A
1 2 3 4 5 6
B
1 2 3 4 5 6
42
38
36

CHAPTER 5: IDENTIFICATION OF PROMOTER ELEMENTS CONCERNED WITH THE UV/BLUE LIGHT INDUCTION OF *CHS*

5.1 Introduction

In addition to studying UV/blue light signal transduction, it is important to define the regulatory *cis*-elements concerned with the transcriptional activation of *CHS*. The transcription factors that interact with these elements will be the 'targets' of the signalling pathways. For this analysis, a UV/blue light inducible transient expression system in *Arabidopsis* protoplasts was developed. By examining the expression of *CHS* promoter-reporter fusions, it is shown that a single light regulatory element in the *Arabidopsis* promoter is both necessary and sufficient for the UV-B and UV-A/blue light induction of *CHS*. Subsequently, the transient expression system was used to study signal transduction involved in the UV-B and UV-A/blue light induction of *CHS* promoter activity.

5.2 UV-B and UV-A/blue Light Induce *CHS* transcript Accumulation in *Arabidopsis* Protoplasts

Parsley protoplasts retain the responsiveness of previously dark-grown, suspension-cultured cells to UV light (Dangl *et al.*, 1987). Thus, chimaeric gene fusions have been introduced into parsley protoplasts to analyse the effects of promoter manipulations on the light-dependent expression of a reporter gene, such as the *uidA* gene encoding β-glucuronidase (GUS) (Jefferson *et al.*, 1986). *CHS* promoter function can then easily be detected by measuring specific GUS activity. A similar approach was used to study *CHS* promoter activity in protoplasts isolated from *Arabidopsis* cells. Figure 5.1A shows that protoplasts can readily be obtained from the *Arabidopsis* cell culture by an overnight treatment with the cell wall degrading enzymes, cellulase and macerozyme.

Moreover, these protoplasts were routinely found to be of high viability (almost 100%) after staining with fluorescein diacetate (data not shown).

Prior to transient expression studies, it was important to establish whether the regulation of *CHS* expression in *Arabidopsis* protoplasts was similar to that observed in the *Arabidopsis* cell culture. Hence protoplasts were transferred to different light qualities for 16 h, total RNA isolated and *CHS* transcripts analysed. Once again, the cDNA probe encoding the *H1* gene product was used as a constitutive control. Figure 5.1B shows that *CHS* transcripts are at a very low level in protoplasts kept in darkness but show a large increase following transfer to UV-B, UV-A/blue light or high intensity white light. This is consistent with the *CHS* expression pattern found in the *Arabidopsis* cell culture (Fig. 3.3). Therefore, *Arabidopsis* cell culture protoplasts provide a suitable system for studying *CHS* promoter function.

5.3 Transient Expression in *Arabidopsis* Protoplasts

Transient expression studies have generally employed the use of electroporation or polyethylene glycol (PEG) to introduce genes into plant protoplasts. Both approaches were used in an attempt to deliver plasmid DNA into *Arabidopsis* cell culture protoplasts. First, transient expression of a choramphenicol acetyltransferase (CAT) reporter gene driven by the constitutively expressed cauliflower mosaic virus 35S promoter was examined following electroporation into cell culture protoplasts. Electroporation was performed at various capacitances and voltages using a method previously shown to support efficient *35S-CAT* expression in *Phaseolus vulgaris* leaf protoplasts (N.A.R. Urwin and G.I. Jenkins, unpublished). Since methylation has been shown to influence reporter gene expression in parsley protoplasts, all plasmid DNA was prepared in the methylation-deficient *E. coli* strain, GM2163 (Torres *et al.*, 1993). A typical experiment is shown in Figure 5.2A: very little CAT activity was observed in *Arabidopsis* protoplasts over a wide range of electroporation conditions. In contrast, high levels of *35S*-driven CAT expression were routinely measured in

Phaseolus vulgaris protoplasts, demonstrating that the lack of expression in *Arabidopsis* protoplasts was not due to poor quality DNA.

Since electroporation was ineffective, we therefore tried a PEG protocol similar to that described for parsley (Lipphardt *et al.*, 1988). The plasmids used here were constructions containing either the *35S* promoter or the full-length *Arabidopsis CHS* promoter fused to the GUS coding region. These chimaeric gene fusions were found to be highly expressed in protoplasts isolated from the dark-grown *Arabidopsis* cell suspension described by Trezzini *et al.* (1993) (data not shown). Similarly, high levels of *35S* promoter activity were detected in protoplasts derived from the light-grown *Arabidopsis* cell culture (Fig. 5.2B). The activities (pmol min^{-1} mg^{-1} protein) are similar to those reported for other transient expression systems (e.g. Weisshaar *et al.*, 1991b; Feldbrügge *et al.*, 1994). Importantly, incubation of protoplasts in a high fluence rate of white light increased *CHS* promoter activity in comparison to dark controls. On the other hand, transfer of protoplasts to high white light had little effect on *35S*-driven GUS activity. Moreover, no GUS activity was observed in protoplasts either not transfected or transfected with a promoterless GUS fusion (data not shown). Taken together, these results demonstrate that PEG transfection permits high levels of transgene expression in *Arabidopsis* cell culture protoplasts. Moreover, light regulation of the *Arabidopsis CHS* promoter is easily detectable in these protoplasts.

5.4 Factors Affecting *CHS* Promoter Activity

Experiments were then undertaken to examine if the level of transgene expression could be improved in the *Arabidopsis* protoplast system. In parsley, protoplasts are routinely isolated from 5 day old suspension cultures (Dangl *et al.*, 1987; Lipphardt *et al.*, 1988; Weisshaar *et al.*, 1991a, 1991b). For all our experiments, *Arabidopsis* cells were used on the third day after subculture. It was therefore examined whether *CHS* promoter activity is affected by cell age. Protoplasts were isolated from 3 and 5 day old suspension cultures and transfected with the *CHS* promoter fusion. As shown in

Figure 5.3A, protoplasts isolated from 3 day old *Arabidopsis* cells support a high level of light-induced GUS activity. However, no further increase in *CHS*-driven GUS expression was observed in protoplasts isolated from 5 day old cells. In fact, light-induced activity is slightly lower in comparison to that observed in protoplasts isolated from 3 day old cells. Based on these results, all subsequent experiments were performed using protoplasts isolated from 3 day old *Arabidopsis* cells.

The concentrations of cellulase and macerozyme used to prepare protoplasts from the *Arabidopsis* cell culture were equivalent to those required to isolate protopasts from leaf *Arabidopsis* tissue (Damm *et al.*, 1989; Abel and Theologis, 1994). Lower concentrations are used to generate protoplasts from parsley cell cultures (Dangl *et al.*, 1987). This prompted us to examine whether a lower concentration of cellulase and macerozyme could be used to isolate protoplasts from the *Arabidopsis* cell culture without affecting *CHS* promoter activity. Four batches of protoplasts were prepared with either the standard enzyme solution (containing 1% cellulase, 0.25% macerozyme), or dilutions of the original enzyme solution, and used for transient gene expression studies. Although protoplast yield decreased at lower enzyme concentrations, viability was unaffected (data not shown). Interestingly, light-induced *CHS* promoter activity was significantly higher in protoplasts obtained from solutions containing low concentrations of cellulase and macerozyme (Fig. 5.3B). Indeed, maximal GUS activity was observed in extracts from protoplasts prepared in a solution of 0.25% cellulase and 0.06% macerozyme, similar to concentrations used to isolate protoplasts from parsley cells (Dangl *et al.*, 1987). Similar effects were also observed on *35S*-driven GUS expression (data not shown). Therefore, these enzyme concentrations were used to isolate protoplasts in all subsequent experiments. Interestingly, the above findings indicate that an unnecessarily high concentration of cellulase and/or macerozyme can reduce transgene expression in plant protoplasts.

5.5 UV-B and UV-A/blue Light Regulate *CHS* Promoter Activity

The *Arabidopsis* transient expression system was then used to examine the effects of different light qualities on *CHS* promoter activity. As shown in Figure 5.4, *CHS* promoter activity is strongly induced by UV-B and UV-A/blue light. High intensity white light is also very effective whereas no induction was observed in response to red light, which is consistent with the lack of phytochrome induction of *CHS* in the *Arabidopsis* cell culture (Fig. 3.3C). None of the light treatments had much effect on *35S* promoter activity. Thus the differential light regulation of *CHS* promoter activity in cell culture protoplasts is very similar to the *CHS* expression pattern found in the *Arabidopsis* cell culture and in leaf tissue (Fig. 3.3). We therefore consider the light-responsiveness of the *CHS* promoter in *Arabidopsis* protoplasts to reliably reflect the pattern of expression found in cells.

As described in 3.11.4, recent studies have shown that UV-A and blue light act synergistically with UV-B to enhance *CHS* promoter activity in light-grown *Arabidopsis* (G. Fuglevand, J.A. Jackson and G.I. Jenkins, unpublished). It was therefore investigated whether a similar pattern of expression could be observed in *Arabidopsis* protoplasts. In contrast to the situation found in plants, no synergistic response was observed on *CHS* promoter activity in cell culture protoplasts. Instead, illumination of protoplasts with UV-B and UV-A/blue light together reduced GUS activity compared to the induction by UV-B and UV-A/blue light alone (Fig. 5.5). However, it is likely that the simultaneous UV-B and UV-A/blue light treatment had a damaging effect on the protoplasts as similar effects were observed on *35S*-driven GUS expression.

5.6 Kinetics of Induction of *CHS* Promoter Activity in Response to Different Light Qualities

Figure 5.6 shows the induction kinetics of *35S* and *CHS* driven GUS expression in cell culture protoplasts in response to UV-B, UV-A/blue light and high intensity white

light over a 24 h period. Following exposure to UV-B and UV-A/blue light, *CHS* promoter activity is detectable within 4 h and steadily increases with time. In high white light, *CHS* promoter activity is clearly visible within 4 h and reaches a maximum after 8 h. The decline in GUS activity after 24 h may be due to damaging effects on protoplast viability, as similar effects were observed on *35S* promoter activity. Nevertheless, these observations correlate well with the induction of *CHS* transcripts by different light qualities in the *Arabidopsis* cell culture (Fig. 3.5).

5.7 Effects of 5' Promoter Deletions on GUS Activity

Studies with a parsley cell culture and parsley plants demonstrate that UV-B and blue light are the principal light qualities regulating *CHS* transcription (Hahlbrock and Scheel, 1989; Ohl *et al.*, 1989; Frohnmeyer *et al.*, 1992). The parsley *CHS* promoter has been analysed both structurally and functionally with respect to UV light-regulation (Schulze-Lefert *et al.*, 1989a, 1989b; Block *et al.*, 1990; Weisshaar *et al.*, 1991a). Four *cis*-acting elements or boxes are assembled within two functional light regulatory units (LRUs). Boxes I and II form LRU1 whereas upstream boxes III and IV form LRU2. Moreover, studies have shown that LRU1 is necessary and sufficient for light-responsiveness (Block *et al.*, 1990; Weisshaar *et al.*, 1991a). A sequence similar to the LRU1 of parsley is present in the promoter of the *Arabidopsis CHS* gene and is shown in Figure 5.7.

In order to identify important light response elements in the *Arabidopsis CHS* promoter, the effects of various 5' deletions on *CHS* promoter activity in the *Arabidopsis* transient expression system were examined. The 5' deletion constructs containing the full-length promoter of the single *Arabidopsis CHS* gene from position –1969 and deletions thereof (–1448 to –64), relative to the transcription start site, are illustrated in Figure 5.8. The corresponding positions of boxes I and II are also indicated. Cell culture protoplasts were transfected with each fusion and transferred to different light qualities. The induced GUS expression driven by the full-length

promoter and different 5' promoter deletions is shown in Figure 5.9A. As demonstrated earlier, the full-length promoter showed induced GUS activity in response to UV-B, UV-A/blue and high intensity white light whereas red light had no effect. The 5' deletions –1448, –672, –336 and –164 show a nearly identical photoregulation to the full-length promoter (Fig. 5.9A and B). Although the quantitative expression levels of constructs –672, –336, –164 are weaker, the data indicate that *cis*-acting elements further 3' of position –164 are able to mediate the complete light regulation of GUS expression. As shown in Figure 5.9B, expression of the fusions –94 and –62 are not significantly light-induced. In fusion –94, box II of the LRU is deleted whereas the entire LRU is removed in fusion –64. Therefore, deletion –164 can be defined as the minimal light-responsive promoter. Moreover, these data suggest that the LRU mediates both the UV-B and UV-A/blue light induction of the *CHS* promoter in *Arabidopsis* protoplasts. These observations are consistent with previous findings from transient expression studies which demonstrate that the LRU1 from the parsley and *Sinapis CHS* promoters act as light response elements (Block *et al.*, 1990; Weisshaar *et al.*, 1991b; Rocholl *et al.*, 1994).

5.8 The *Arabidopsis* LRU is Sufficient for UV-B and UV-A/blue Light-dependent Expression

To investigate whether the LRU of the *Arabidopsis CHS* gene is sufficient to mediate light-induced expression, a construct carrying the LRU as a tetramer (Fig. 5.8) fused to the minimal *35S* promoter region between –46 and +8 (Weisshaar *et al.*, 1991b) was transfected into cell culture protoplasts and then analysed for light-induced GUS activity. As shown in Figure 5.10, the LRU tetramer fusion showed an almost identical photoregulation pattern to the full-length promoter. Thus these results indicate that the LRU is sufficient for the UV-B and UV-A/blue light induction of the *Arabidopsis CHS* promoter.

5.9 Effects of Various Inhibitors on *CHS* Promoter Activity

In addition to analysing promoter function, transient expression has been used to study signal transduction. For example, Sheen (1993) demonstrated, using a maize protoplast system, that the light-induced expression of two photosynthetic gene fusions was specifically suppressed by protein phosphatase inhibitors. It was therefore examined whether the *Arabidopsis* protoplast system could be used to study UV-B and UV-A/blue light signal transduction regulating *CHS* promoter activity. Figure 5.11A shows that the calcium channel blockers, nifedipine and ruthenium red, severely attenuated UV-B and UV-A/blue light-induced *CHS* promoter activity in *Arabidopsis* protoplasts. These findings are consistent with the effects observed at the transcript level in the *Arabidopsis* cell culture (Fig. 4.1). In contrast to the situation found in cells, incubation of protoplasts with EGTA had very little effect on *CHS-GUS* expression. Nevertheless, this provides additional support for the hypothesis that UV/blue phototransduction pathways controlling *CHS* involve an intracellular source of calcium. Addition of W-7 effectively inhibits the UV-B signalling pathway whereas trifluoperazine prevents *CHS* promoter activity in response to both UV-B or UV-A/blue light (Fig. 5.12A), which is again consistent with the effects observed on *CHS* expression in *Arabidopsis* cells. Similarly, staurosporine, okadaic acid and cantharidin inhibit the UV-B and UV-A/blue light induction of *CHS* promoter activity at concentrations shown to inhibit *CHS* transcript accumulation in cells (Fig. 5.13A). However, with most compounds, almost identical inhibitory effects were observed on *35S*-driven GUS activity (Fig. 5.11B, 5.12B and 5.13B). These findings imply that the inhibitors have a general effect on transcription and/or translation activities in *Arabidopsis* protoplasts. Therefore, unlike the cell suspension system, protoplast transient expression may not be suitable for pharmacological studies investigating signal transduction concerned with promoter activation.

5.10 *CHS* Promoter Activity in *Arabidopsis* Leaf Protoplasts

Transient gene expression has also been reported in *Arabidopsis* leaf protoplasts (Damm *et al.*, 1989; Abel and Theologis, 1994). With a view to extending our studies to mutant backgrounds, *CHS* promoter activity in protoplasts isolated from *Arabidopsis* leaf tissue was examined. Although several protoplast isolation methods were tested (Saxena *et al.*, 1985; Damm *et al.*, 1989; Masson and Paszkowski, 1992; Abel and Theologis, 1994), a protocol obtained from Dr Jen Sheen (Department of Molecular Biology, Massachusetts General Hospital, Boston) was found to be the most effective (recently described in Matsui *et al.*, 1995). As shown in Figure 5.14, viable leaf protoplasts can be prepared in this way from 21 day old light-grown *Arabidopsis* leaves. Freshly prepared leaf protoplasts were then used for transient expression studies. First of all, the possiblilty of using electroporation was examined. Similar to the situation found in cell culture protoplasts, very little CAT activity was detected in *Arabidopsis* leaf protoplasts over a wide range of conditions (Fig. 5.15A). As a control, high levels of CAT activity were observed in *Phaseolus vulgaris* leaf protoplasts electroporated with the same gene fusion. On the other hand, *35S*-driven GUS expression was strongly induced in leaf protoplasts transfected using the PEG protocol (Fig. 5.15B). Moreover, a significant amount of UV-A/blue light-induced *CHS* promoter activity was observed in these protoplasts. Using this procedure it should then be possible to study *CHS* promoter activity in leaf protoplasts isolated from various *Arabidopsis* mutants. However, as discussed above, these protoplasts are unlikely to be useful for signal transduction studies.

5.11 Discussion

In the present chapter, the development of an *Arabidopsis* transient expression system to investigate *cis*-regulatory elements concerned with the UV-B and UV-A/blue light induction of *CHS* is described. The approach used here is very similar to the parsley protoplast system that has provided information on the *cis*-elements and transcription

factors concerned with the photoregulation of *CHS*. From transient expression studies with cell culture protoplasts, we have identified a single *cis*-acting light regulatory unit (LRU) within the *Arabidopsis CHS* promoter with high similarity to LRU1 of the parsley *CHS* gene. Further analysis shows that the LRU of the *Arabidopsis CHS* gene is both necessary and sufficient to mediate responses to UV-B and UV-A/blue light.

5.11.1 Transient expression in *Arabidopsis* protoplasts

Within the last decade, *Arabidopsis* has become the most widely used model system for plant molecular biology (Meyerovitz, 1989). The small genome size, powerful genetics and relatively simple organisation has made gene cloning easier compared to other plant species. Indeed, many genes have been isolated and characterised. Studies of gene expression have been undertaken primarily in stably transformed *Arabidopsis* plants or in other easily transformed plants, such as tobacco. Although necessary to investigate tissue specificity and developmental regulation, the production of transgenic plants is a time consuming process. Transient expression of genes in plant protoplasts is a much faster way of analysing essential *cis*-acting sequences and has been used extensively in established plant systems such as parsley (e.g. Lipphardt *et al.*, 1988; Weisshaar *et al.*, 1991a). More recently, several protocols have been published describing transient gene expression in *Arabidopsis* (Damm *et al.*, 1989; Axelos *et al.*, 1992; Doelling and Pikaard, 1993; Abel and Theologis, 1994). In these reports, the polyvalent cation, PEG, was used to introduce DNA into *Arabidopsis* protoplasts. PEG is thought to precipitate DNA molecules, reduce charge repulsion between the DNA and the plasma membrane and thereby promote DNA uptake by endocytosis. A similar procedure was used in this study to express chimaeric gene fusions in *Arabidopsis* protoplasts. The level of transgene expression varied between experiments and was probably due to differences in transfection efficiency or protoplast quality. Therefore, the data presented are from individual experiments which are representative of the results obtained.

Rapidly growing *Arabidopsis* cells yield excellent protoplasts which support efficient transient expression of a GUS reporter gene driven by the *35S* promoter (Fig. 5.2B). Similar observations were observed using protoplasts isolated from *Arabidopsis* leaf tissue (Fig. 5.15B). However, in our hands, leaf protoplasts appear to be very fragile compared to those derived from the *Arabidopsis* cell culture. Moreover, the quantity and quality of protoplasts prepared from leaf tissue varies greatly from batch to batch, even with plants grown in the same environment. In contrast, large amounts of viable protoplasts can reliably be obtained from the *Arabidopsis* cell culture, thereby circumventing some of the problems resulting from the small size of *Arabidopsis*. Electrical pulses have been used to reversibly permeabilise the plant cell membrane, allowing the uptake of DNA for transient expression studies (e.g. Dron *et al.*, 1988; Dekeyser *et al.*, 1990; Sheen, 1993). While the PEG transfection procedure described here works well with *Arabidopsis* leaf and cell culture protoplasts, no significant level of transgene expression was observed over a wide range of electroporation conditions (Fig. 5.2A and Fig. 5.15A). In each case, the lack of CAT activity was not due a defect in the DNA itself since high levels of *35S*-driven CAT expression were measured in protoplasts derived from *Phaseolus vulgaris* primary leaves. Similarly, no GUS activity was observed in *Arabidopsis* protoplasts electroporated with *35S-GUS*, demonstrating that the effectiveness of PEG over electroporation was not due to a difference in reporter genes (data not shown). While electroporation has been used to deliver DNA into intact rice cells (Dekeyser *et al.*, 1990) and *Phaseolus vulgaris* tissues (N.A.R. Urwin and G.I. Jenkins, unpublished), no significant GUS activity was observed in *Arabidopsis* cells electroporated with the *35S* promoter fusion (data not shown). It is likely that the lack of expression results from adverse effects of electroporation on cell viability. Another possiblity is that, under the conditions examined, electroporation was not sufficient to permeabilise the *Arabidopsis* plasma membrane. If so, the appropriate electroporation conditions required for nucleic acid uptake into *Arabidopsis* cells and protoplasts have yet to be determined.

5.11.2 *Arabidopsis* protoplasts retain responsiveness to UV-B and UV-A/blue light

Consistent with the *CHS* expression pattern found in cells, *CHS* transcript accumulation is regulated by distinct UV-B and UV-A/blue phototransduction pathways in *Arabidopsis* cell culture protoplasts (Fig. 5.1B). Furthermore, the full-length promoter of the *Arabidopsis CHS* gene is sufficient for UV-B and UV-A/blue light-induced GUS expression (Fig. 5.4). Transient expression in *Arabidopsis* protoplasts therefore provides an excellent system for the analysis of *cis*-acting elements concerned with the UV-B and UV-A/blue light regulation of *CHS*. Interestingly, lowering the concentration of cellulase and macerozyme required for the preparation of cell culture protoplasts increased the quantitative expression of *CHS* by approximately 10-fold (Fig. 5.3B). These observations may result from damaging effects of contaminants, such as proteases, present in the crude enzyme preparations. In support of this hypothesis, some protoplast isolation protocols heat treat the digestion solution to inactivate proteases (J. Sheen, personal communication). Indeed, Somerville *et al.* (1981) found it necessary to purify the enzyme solution through a sephadex column to obtain active chloroplasts from *Arabidopsis* protoplasts. Thus removal or dilution of potentially harmful factors from the cell wall digestion soultion appears to be a necessary prerequisite for efficient transient gene expression in plant protoplasts.

The full-length promoter of the *Arabidopsis CHS* gene is also sufficient for light-induced GUS expression in *Arabidopsis* leaf protoplasts (Fig. 5.15B). An advantage of a leaf protoplast system is that gene expression studies can be extended to mutant backgrounds. However, in leaves, *CHS* expression is restricted to the epidermal layer (Schmelzer *et al.*, 1988; Chory and Peto, 1990). As leaves mostly consist of mesophyll cells, a leaf protoplast transient expression system may not be suitable for studying *CHS* promoter function. Indeed, the relatively small level of light induction observed in our experiments (about 6-fold) may result from *CHS*-driven GUS expression in transfected epidermal protoplasts. Despite this, the *Arabidopsis* leaf protoplast system may be useful for studying other light-regulated genes, such as *CAB*

or *rbcS*. Nevertheless, the experiments with pharmacological reagents (Fig. 5.11, 5.12 and 5.13) indicate that protoplasts are less suitable for signal transduction studies than cultured cells, so the value of using leaf protoplasts will be limited.

5.11.3 The LRU mediates the effects of UV-B and UV-A/blue light on *CHS* expression

Transient expression assays with chimaeric *CHS* promoter fusions have identified *cis*-acting sequences functioning as light regulatory units or LRUs (Schulze-Lefert *et al.*, 1991a, 1991b; Weisshaar *et al.*, 1991a, 1991b; Rocholl *et al.*, 1994). One of these, LRU1 was primarily identified by *in vivo* footprinting analysis in parsley and contains two binding sites for protein interactions: box II, a member of the family of ACGT elements and box I (Schulze-Lefert *et al.*, 1991a). Box II, similar to the G-box of *rbcS* and other promoters (Menkens *et al.*, 1995), binds strongly to bZIP proteins *in vitro* (Weisshaar *et al.*, 1991a; Feldbrügge *et al.*, 1994), whereas the binding activity of box I appears to belong to the *myb* class of transcription factors (Hahlbrock *et al.*, 1995). Both box I and box II are required for light induction, because mutations of specific nucleotides within either of these elements leads to a loss of function (Block *et al.*, 1990). Further studies demonstrate that the LRU1 from parsley is able to confer light-responsiveness on a heterologous transcription start site (Weisshaar *et al.*, 1991a, 1991b). Therefore, LRU1 is not only necessary but sufficient for directing light-dependent expression in parsley protoplasts. Upstream boxes III and IV form an additional light regulatory unit (LRU2) which can also mediate light-regulated expression in the absence of LRU1, although LRU1 is more effective (Schulze-Lefert *et al.*, 1989b).

The results presented here show that a sequence in the *Arabidopsis CHS* promoter corresponding to the LRU1 of parsley is sufficient for light-induced expression (Fig. 5.9 and Fig. 5.10). Furthermore, both the UV-B and UV-A/blue light regulation of *CHS* promoter activity is mediated by the LRU. Thus it is unlikely that

UV-B and UV-A/blue light exert their effects on *CHS* expression via functionally separate *cis*-acting regulatory units. Similarly, Merkle *et al.* (1994) observed that UV-B and blue light lead to the same previously observed light-induced *in vivo* footprint pattern of the parsley *CHS* promoter (Schulze-Lefert *et al.*, 1989a, 1989b). More recently, the LRU1 from the *Sinapis CHS1* gene has been shown to be sufficient for UV/blue light-induced promoter activity in transgenic *Arabidopsis* and tobacco (Kaiser *et al.*, 1995).

Although the LRU of the *Arabidopsis CHS* promoter responds to different light qualities in the same way as the full-length promoter, it is possible that other regions of this promoter modulate expression levels. Indeed, the LRU within the minimal promoter (construct –164) generates only about 10% of the maximal GUS activity driven by the full-length promoter (Fig. 5.9). Deletion analysis suggests that sequences upstream of –672 influence the level of light-activated GUS expression. However, it is worth noting that, in separate experiments, the drop in GUS activity was not always apparent at position –672. While a similar sequence to LRU2 is present in the *Sinapis CHS1* gene (Kaiser and Batschauer, 1995), no upstream homologies to boxes III and IV have been identified in *Arabidopsis*. Nevertheless, it will be important to identify the upstream sequences that enhance the light-responsivenss of the minimal promoter in *Arabidopsis*. Interestingly, the presence of a region upstream of LRU2 in the parsley *CHS* promoter has been found to enhance the quantitative expression mediated by both light regulatory units (Schulze-Lefert *et al.*, 1989b).

5.11.4 Analysis of UV-B and UV-A/blue light signal transduction using transient expression

Transient gene expression presents several advantages over northern analysis for studying signal transduction processes concerned with gene expression. Firstly, the protoplast system offers a more rapid means to measure the transcription of specific genes. Secondly, constitutively expressed promoter fusions, such as *35S-GUS*, can

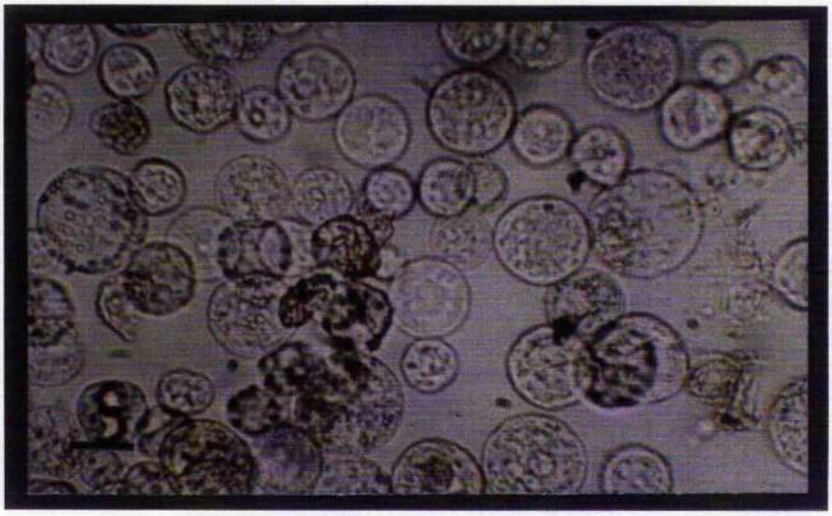
act as a control to determine whether signalling agonists/antagonists affect transcription in general. Furthermore, the effects of various signalling reagents on defined promoter elements can be investigated. Despite these advantages, our results suggest that the *Arabidopsis* transient expression system may not be suitable for studying UV-B and UV-A/blue light signal transduction regulating *CHS*. In contrast to their specific effects in cells, many of the pharmacologal agents caused a general inhibition of gene expression in *Arabidopsis* protoplasts. Therefore, it seems likely that these compounds are toxic to protoplasts at these concentrations. Consistent with this, expression of GUS driven by an *Arabidopsis* polyubiquitin promoter was also reduced by these reagents (data not shown). It is worth investigating whether such effects could be reduced by either lowering the inhibitor concentration or reducing the exposure time of protoplasts to the inhibitors. However, not all of the compounds used in this chapter were detrimental to the cell culture protoplasts. For instance, the differential effect of W-7 on the UV-B and UV-A/blue light induction of *CHS* promoter activity is consistent with the effects observed on *CHS* transcript accumulation in the *Arabidopsis* cell culture (Fig. 5.12A). Moreover, the effects of staurosporine on *CHS* promoter activity appear to be specific since *35S*-driven GUS expression is less affected (Fig. 5.13A). Incubation of protoplasts with 10 mM EGTA had no effect on either *CHS* or *35S*-driven GUS expression (Fig. 5.11A), supporting the hypothesis that extracellular calcium is not involved in the UV-B and UV-A/blue light signalling pathways. However, we previously observed that EGTA, at equivalent concentrations, strongly inhibits the accumulation of *CHS* transcripts by UV-B and UV-A/blue light in the *Arabidopsis* cell culture. A possible explanation for this discrepancy is that a rapid depletion of cell wall calcium somehow results in a general inhibition of transcriptional activation. Such an effect would not be observed in cell culture protoplasts. Taken together, these findings strongly suggest that pharmacological studies with transient gene expression should be interpreted with caution without the appropriate controls.

Figure 5.1. UV-B and UV-A/blue light induce *CHS* transcript accumulation in *Arabidopsis* cell culture protoplasts

(A) Photograph of protoplasts isolated from the *Arabidopsis* cell culture viewed under a microscope. The size bar represents approximately 30 μm.

(B) *Arabidopsis* cells grown in 20 μmol m^{-2} s^{-1} white light were used to generate cell culture protoplasts. Protoplasts were isolated in a low fluence rate of white light (5-10 μmol m^{-2} s^{-1}) and transferred to the following different light qualities for 20 h: dark (lane 1), 100 μmol m^{-2} s^{-1} white light (lane 2), 50 μmol m^{-2} s^{-1} UV-A/blue light (lane 3) or 1.5 μmol m^{-2} s^{-1} UV-B (lane 4). Total RNA was isolated, 10 μg separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. *CHS* transcript levels were measured using an *Arabidopsis CHS* probe and the membrane stripped of radioactivity before rehybridising to the *Phaseolus vulgaris H1* probe.

A



B

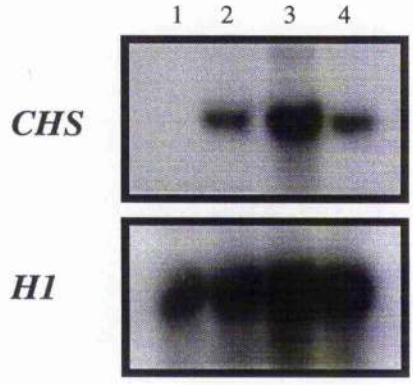


Figure 5.2. Electroporation and PEG-stimulated DNA uptake into *Arabidopsis* cell culture protoplasts

(A) Protoplasts isolated from the *Arabidopsis* cell culture and *Phaseolus vulgaris* primary leaves were electroporated, under the conditions indicated, with 25 μg of the *35S-CAT* fusion. Following transfection, protoplasts were incubated in 20 μmol m^{-2} s^{-1} white light for 20 h, harvested and CAT activity (cpm) determined.

(B) Cell culture protoplasts were transfected with 20 μg of either the *35S-GUS* or *CHS-GUS* fusion using PEG and transferred to darkness (D) or 100 μmol m^{-2} s^{-1} white light (L) for 20 h. Following incubation, protoplasts were harvested and assayed for GUS activity. Values are the means of 4 (*35S*) and 6 (*CHS*) different samples. Error bars represent the SD.

A

Protoplasts	Electroporation prameters	Relative CAT activity (cpm)
Phaseolus vulgaris leaf protoplasts	100 μF, 350 V	27,492
Arabidopsis cell culture protoplasts	21 μF, 250 V	17
	21 μF, 500 V	103
	21 μF, 750 V	0
	21 μF, 1000 V	0
	21 μF, 1250 V	0
	100 μF, 100 V	16
	100 μF, 200 V	289
	100 μF, 300 V	0
	100 μF, 400 V	0
	100 μF, 500 V	2

B

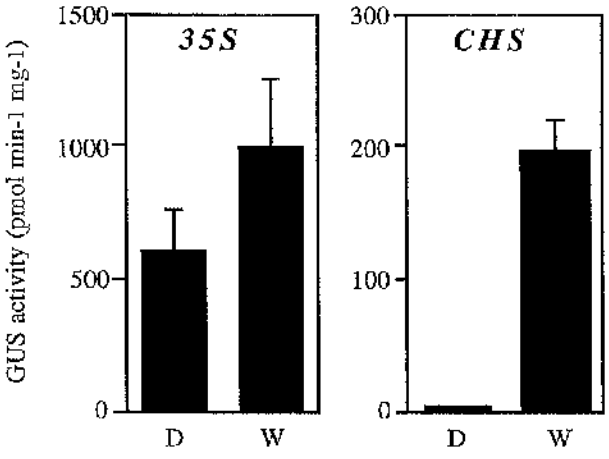
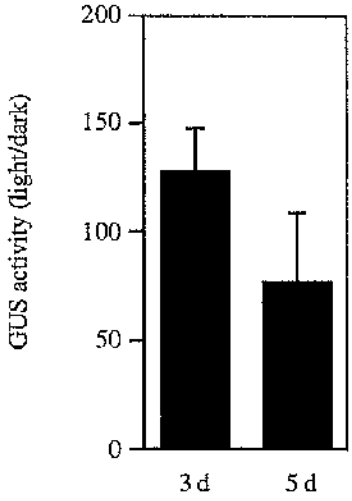


Figure 5.3. Effects of cell age and cell wall degrading enzyme concentration on *CHS* promoter activity

(A) Protoplasts isolated from 3 day (3 d) or 5 day (5 d) old *Arabidopsis* cells were transfected with 20 µg of the *CHS-GUS* fusion using PEG and transferred to darkness or 100 µmol m^{-2} s^{-1} white light for 20 h. After incubation, protoplasts were harvested and GUS activity determined. Induction ratios (light/dark) are shown. Values are the means of duplicate samples. Error bars represent the SD. Dark levels (pmol min^{-1} mg^{-1} protein ± SD) were as follows: 4.4 ± 0.1 for 3 d and 6.8 ± 0.8 for 5 d.

(B) Four different protoplast suspensions were isolated from *Arabidopsis* cells using either the standard enzyme solution (100%), 1/2 dilution of the standard enzyme solution in 0.24 M $CaCl_2$ (50%), 1/4 dilution (25%) or a 1/10 dilution (10%). Protoplasts from each suspension were transfected prior to incubation as in (A) and GUS activity determined. Error bars indicate the SD. Dark levels (pmol min^{-1} mg^{-1} protein ± SD) were as follows: 8.6 ± 3.5 for 100%, 3.7 ± 0.3 for 50%, 5.9 ± 2.4 for 25% and 6.5 ± 2.5 for 10%.

A


GUS activity (light/dark)
200
150
100
50
0
3 d
5 d

B

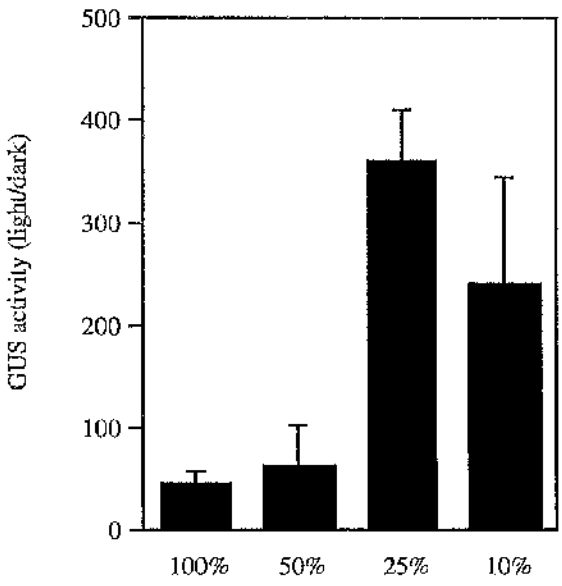

GUS activity (light/dark)
500
400
300
200
100
0
100%
50%
25%
10%

Figure 5.4. UV-B and UV-A/blue light induce *CHS* promoter activity

Cell culture protoplasts were transfected with 20 μg of either the *35S-GUS* or *CHS-GUS* fusion using PEG and transferred to the following different light qualities: dark (D), 100 μmol m^{-2} s^{-1} white (W), 50 μmol m^{-2} s^{-1} red (R), 50 μmol m^{-2} s^{-1} UV-A/blue (B) and 1.5 μmol m^{-2} s^{-1} UV-B. Protoplasts were harvested after 20 h and assayed for GUS activity. Values are the means of duplicate samples. Errors bars represent the SD.

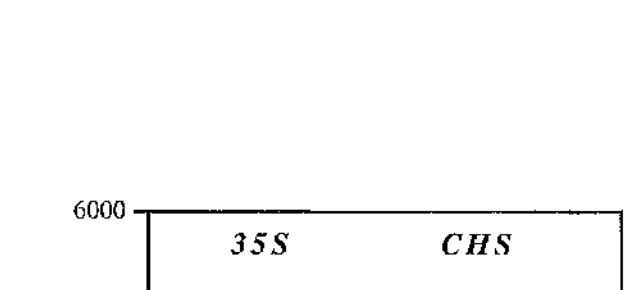

6000
4000
2000
0
GUS activity (pmol min-1 mg-1)
35S
CHS
D W R B UVB D W R B UVB

Figure 5.5. Effects of a simultaneous UV-B and UV-A/blue light treatment on *CHS* promoter activity

Cell culture protoplasts were transfected with 20 μg of either the *35S-GUS* or *CHS-GUS* fusion using PEG and transferred to the following different light qualities: 50 μmol m^{-2} s^{-1} UV-A/blue light (B), 1.5 μmol m^{-2} s^{-1} UV-B, or UV-A/blue and UV-B (at the above fluence rates). Protoplasts were harvested after 8 h and GUS activity determined. Values represent the average of duplicate samples. Error bars are the SD.

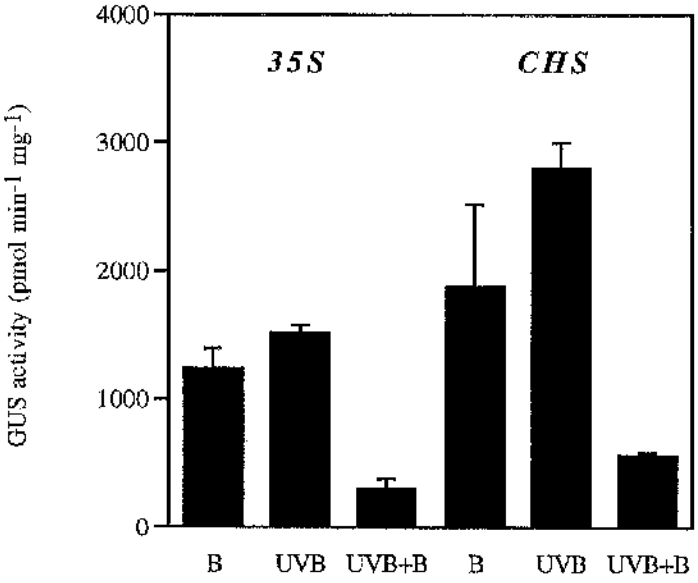

4000
3000
2000
1000
0
GUS activity (pmol min-1 mg-1)
35S
CHS
B
UVB
UVB+B
B
UVB
UVB+B

Figure 5.6. Kinetics of Induction of *CHS* promoter activity in response to different light qualities

Cell culture protoplasts were transfected with 20 µg of either the *35S-GUS* or *CHS-GUS* fusion using PEG and transferred to **(A)** 50 µmol m^{-2} s^{-1} UV-A/blue light, **(B)** 1.5 µmol m^{-2} s^{-1} UV-B or **(C)** 100 µmol m^{-2} s^{-1} white light for the times indicated (h). Protoplasts were harvested and assayed for GUS activity. Values are the means of duplicate samples. Error bars indicate the SD.

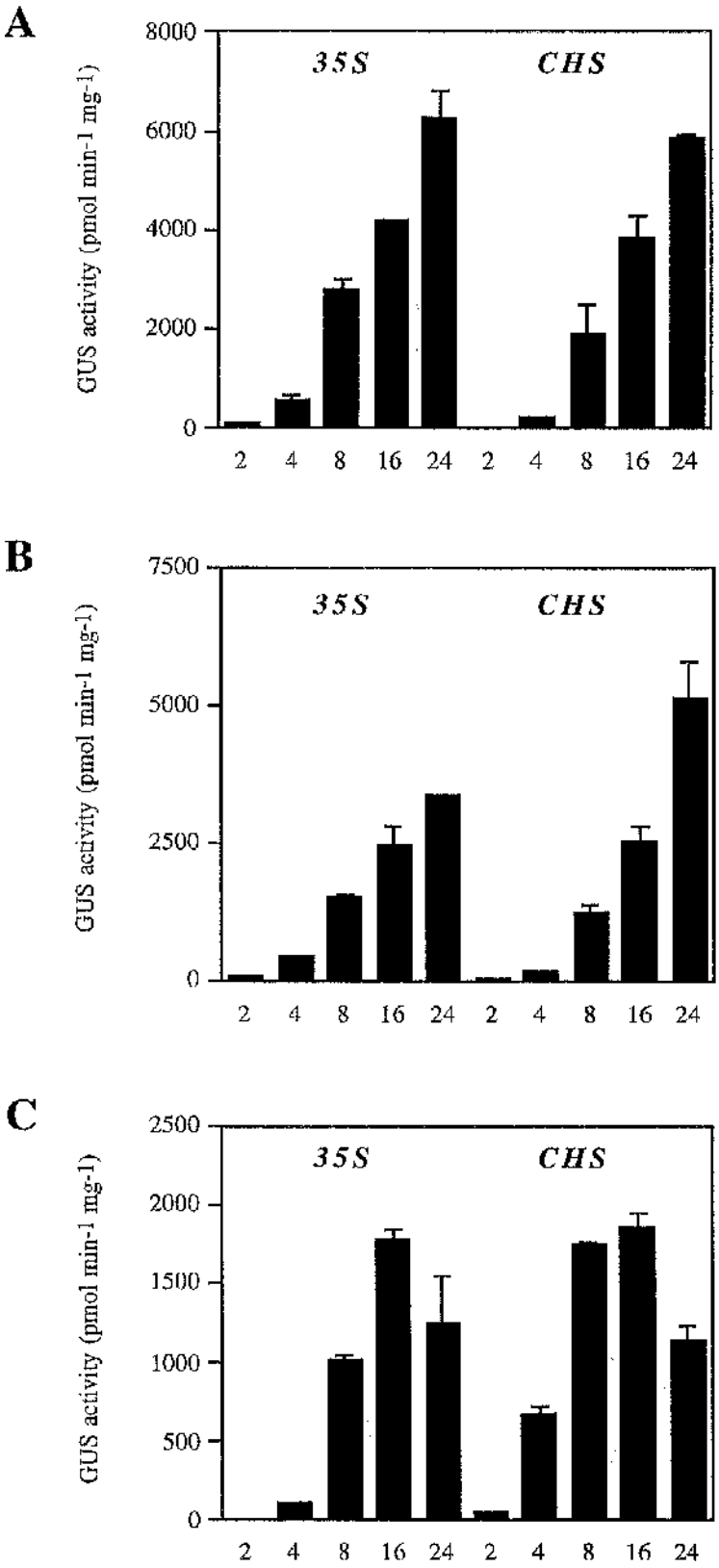

A
B
C
35S
CHS
GUS activity (pmol min-1 mg-1)
8000
6000
4000
2000
0
7500
5000
2500
1500
1000
500
2 4 8 16 24 2 4 8 16 24

Figure 5.7. Sequence comparison of LRU1 of the parsley *CHS* promoter with corresponding sequences in the *CHS* promoter from *Arabidopsis*

The numbers indicate the distance (bp) relative to the transcription start site of the original genes. The number of nucleotides between the ACGT motif (box II) and box I in each promoter is shown. Vertical lines indicate sequence identity.

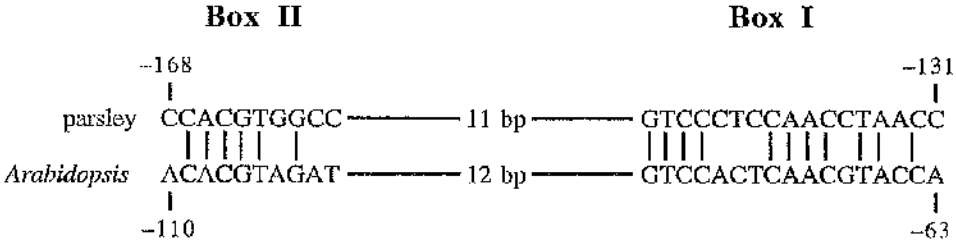
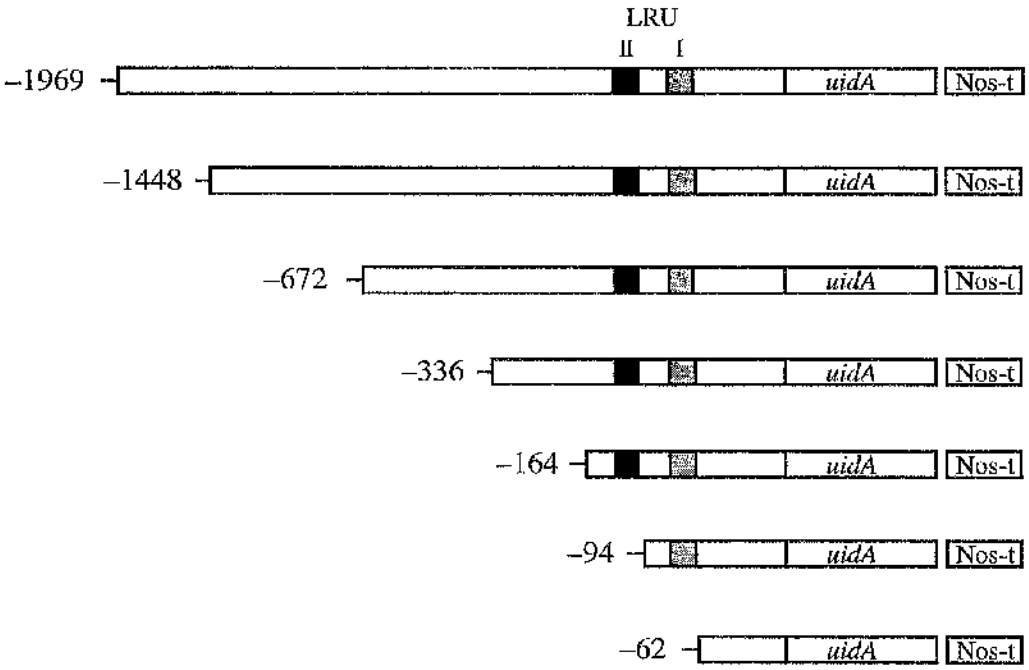

Box II
Box I
−168
−131
parsley
CCACGTGGCC
11 bp
GTCCCTCCAACCTAACC
Arabidopsis
ACACGTAGAT
12 bp
GTCCACTCAACGTACCA
−110
−63

Figure 5.8. Schematic represention of the *CHS* promoter fusions used for promoter analysis

All 5' deletions are fused to the *uidA* open reading frame encoding GUS. The fusions have a common nopaline synthase gene 3' untranslated sequence at the 3' end (Nos-t) and varying 5' ends spanning from position –1969 to –62 of the *Arabidopsis CHS* promoter. Boxes I and II of the light response unit (LRU) are indicated. The LRU tetramer construct contains four copies of the LRU of the *Arabidopsis CHS* gene in the original orientation fused to the –46 deletion of the *35S* promoter. The numbers indicate the distance (bp) relative to the transcription start site of the genes.

5' Deletions

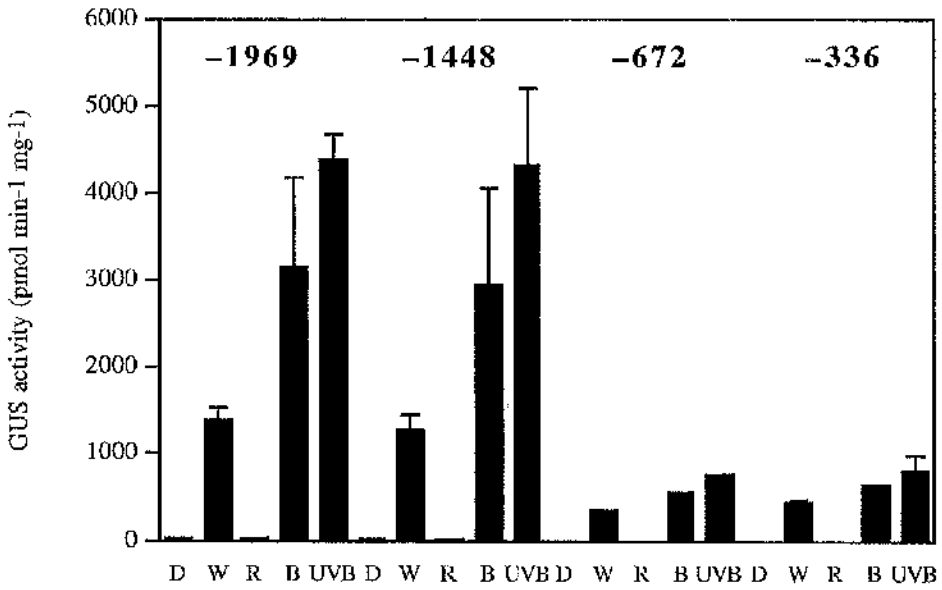


LRU tetramer

Figure 5.9. Light-regulated expression of *CHS* promoter deletion fusions

Cell culture protoplasts were transfected with 20 µg of each of the 5' promoter deletion fusions **(A)** –1969, –1448, –672 and –336 or **(B)** –164, –94 and –62 using PEG. Transfected protoplasts were transferred to the following different light qualities for 20 h: dark (D), 100 µmol m^{-2} s^{-1} white (W), 50 µmol m^{-2} s^{-1} red (R), 50 µmol m^{-2} s^{-1} UV-A/blue (B) and 1.5 µmol m^{-2} s^{-1} UV-B. Following incubation, protoplasts were harvested and GUS activity determined. Values are the means of duplicate samples. Error bars indicate the SD.

A


GUS activity (pmol min-1 mg-1)
6000
5000
4000
3000
2000
1000
0
-1969
-1448
-672
-336
D W R B UVB D W R B UVB D W R B UVB D W R B UVB

B

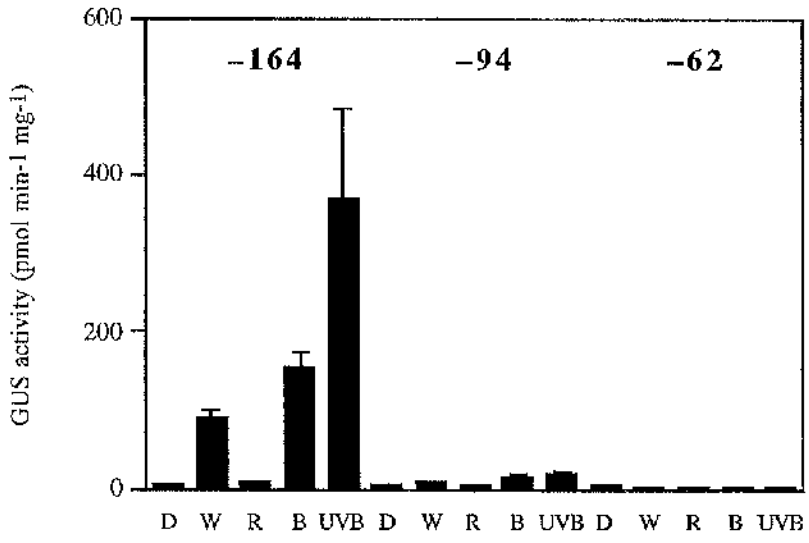

GUS activity (pmol min-1 mg-1)
600
400
200
0
-164
-94
-62
D W R B UVB D W R B UVB D W R B UVB

Figure 5.10. LRU-driven GUS expression in response to different light qualities

Cell culture protoplasts were transfected with 20 μg of the LRU tetramer-truncated *35S* promoter fusion (Fig. 5.8) using PEG. Protoplasts were transferred to the following different light qualities for 20 h: dark (D), 100 μmol m^{-2} s^{-1} white (W), 50 μmol m^{-2} s^{-1} red (R), 50 μmol m^{-2} s^{-1} UV-A/blue (B) and 1.5 μmol m^{-2} s^{-1} UV-B. After incubation, protoplasts were harvested and assayed for GUS activity. Values are the means of duplicates. Error bars represent the SD.

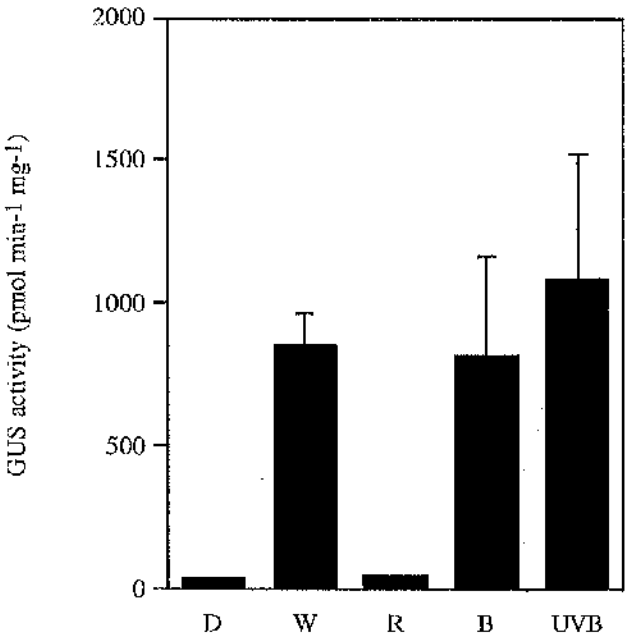

GUS activity (pmol min-1 mg-1)
2000
1500
1000
500
0
D
W
R
B
UVB

Figure 5.11. Effects of calcium antagonists on UV-B and UV-A/blue light-induced *CHS* promoter activity

Cell culture protoplasts transfected with **(A)** 20 μg of the *CHS-GUS* fusion or **(B)** 20 μg of the *35S-GUS* fusion were incubated for 30 min in darkness either without (–) or with 10 mM EGTA, 50 μM nifedipine (NIF) or 50 μM ruthenium red (RR) prior to illumination with UV-B (1.5 μmol m^{-2} s^{-1}) or UV-A/blue light (50 μmol m^{-2} s^{-1}). Protoplasts were harvested after 8 h and GUS activity determined. Values are relative to the controls without inhibitor treatment (–) and represent the average of duplicate samples. Error bars indicate the SD. The dark levels of *CHS-GUS* and *35S-GUS* expression (pmol min^{-1} mg^{-1} protein ± SD) were 19.9 ± 3.0 and 498.2 ± 72.3 respectively.

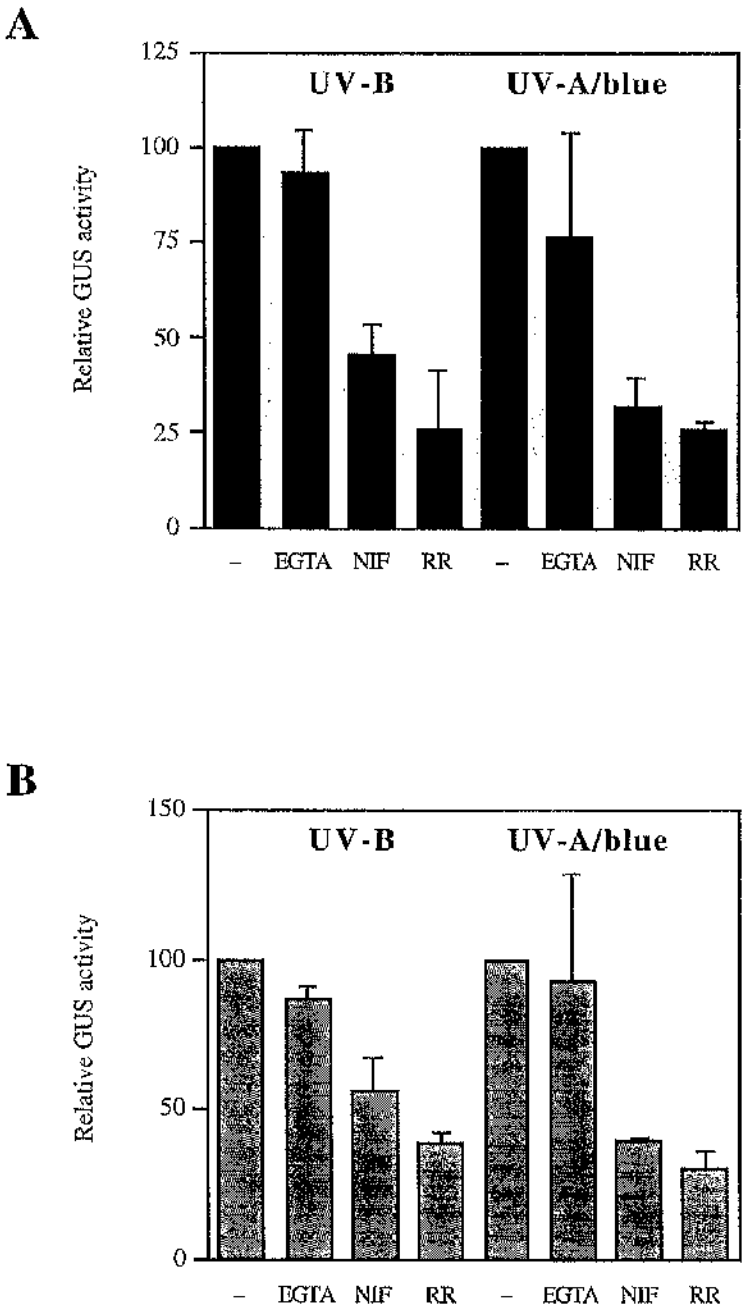

A
125
100
75
50
25
0
Relative GUS activity
UV-B
UV-A/blue
–
EGTA
NIF
RR
–
EGTA
NIF
RR
B
150
100
50
0
Relative GUS activity
UV-B
UV-A/blue
–
EGTA
NIF
RR
–
EGTA
NIF
RR

Figure 5.12. Effects of calmodulin antagonists on UV-B and UV-A/blue light-induced *CHS* promoter activity

Cell culture protoplasts transfected with **(A)** 20 μg of the *CHS-GUS* fusion or **(B)** 20 μg of the *35S-GUS* fusion were incubated for 30 min in darkness either without (–) or with 25 μM W-7 or 25 μM trifluoperazine (TFP) prior to illumination with UV-B (1.5 μmol m^{-2} s^{-1}) or UV-A/blue light (50 μmol m^{-2} s^{-1}). Protoplasts were harvested after 8h and GUS activity determined. Values shown are relative to the controls without inhibitor treatment (–) and represent the average of duplicate samples. Error bars indicate the SD. The dark levels of *CHS-GUS* and *35S-GUS* expression (pmol min^{-1} mg^{-1} protein ± SD) were 7.3 ± 1.1 and 377.0 ± 45.5 respectively.

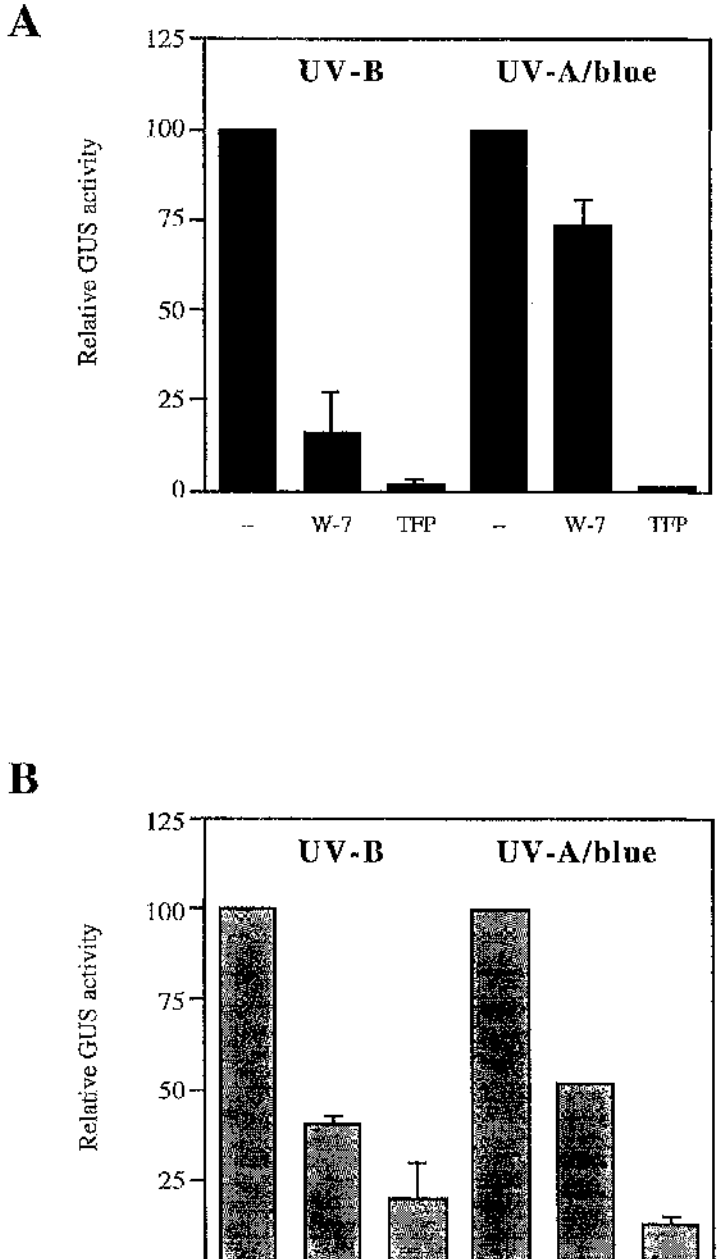
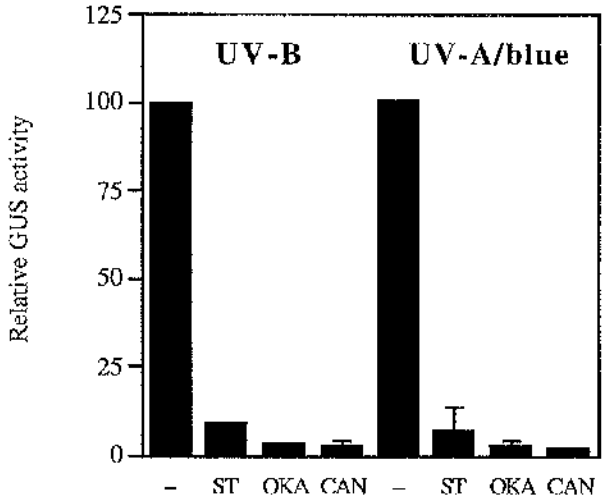

A
B
UV-B
UV-A/blue
Relative GUS activity
125
100
75
50
25
0
–
W-7
TFP

Figure 5.13. Effects of protein kinase and phosphatase inhibitors on UV-B and UV-A/blue light-induced *CHS* promoter activity

Cell culture protoplasts transfected with **(A)** 20 µg of the *CHS-GUS* fusion or **(B)** 20 µg of the *35S-GUS* fusion were incubated in darkness for 30 min either without (–) or with 1 µM staurosporine (ST), 1 µM okadaic acid (OKA) or 1 µM cantharidin (CAN) prior to illumination with UV-B (1.5 µmol m^{-2} s^{-1}) or UV-A/blue light (50 µmol m^{-2} s^{-1}). Protoplasts were harvested after 8 h and GUS activity determined. Values are relative to the controls without inhibitor treatment (–) and represent the average of duplicate samples. Error bars indicate the SD. The dark levels of *CHS-GUS* and *35S-GUS* expression (pmol min^{-1} mg^{-1} protein ± SD) were 3.2 ± 0.5 and 290.0 ± 8.6 respectively.

A


Relative GUS activity
125
100
75
50
25
0
UV-B
UV-A/blue
–
ST
OKA
CAN
–
ST
OKA
CAN

B

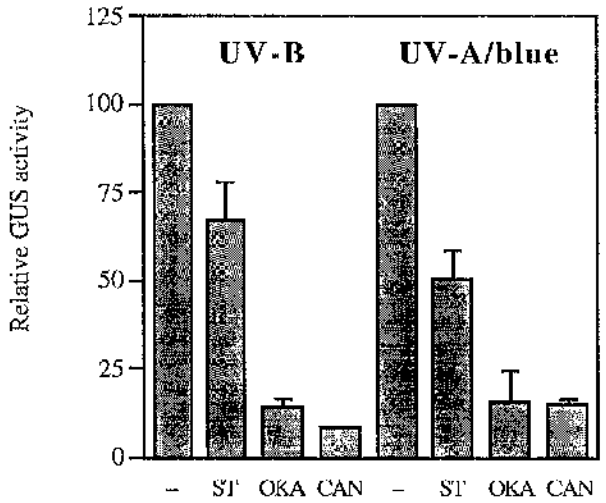
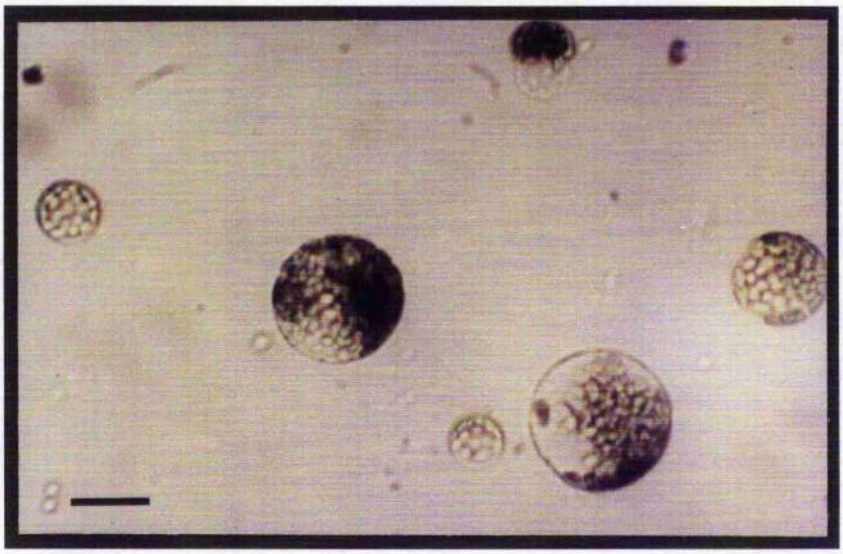

Relative GUS activity
125
100
75
50
25
0
UV-B
UV-A/blue
–
ST
OKA
CAN
–
ST
OKA
CAN

Figure 5.14. Photographs of *Arabidopsis* leaf protoplasts

(A) Protoplasts isolated from *Arabidopsis* leaf tissue viewed under a microscope. The size bar represents approximately 30 μm.

(B) Protoplasts shown in (A) stained with fluorescein diacetate viewed under a fluorescence microscope. Live protoplasts stain green whereas non-viable protoplasts stain red.

A



B

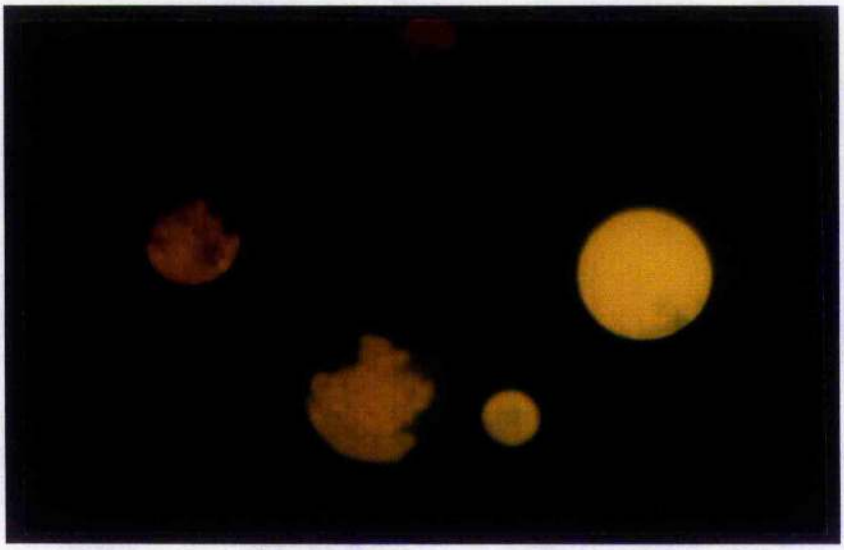


Figure 5.15. Electroporation and PEG-stimulated DNA uptake into *Arabidopsis* leaf protoplasts

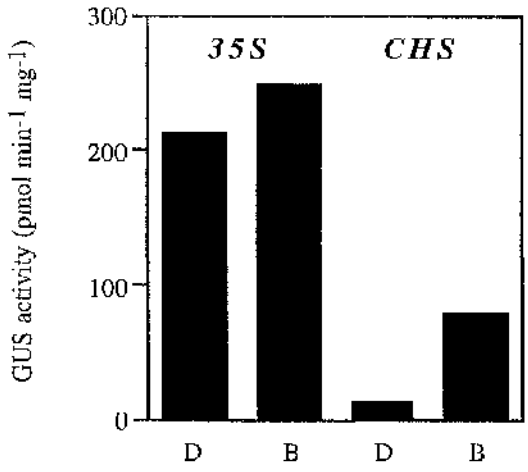
(A) Leaf protoplasts isolated from *Arabidopsis* and *Phaseolus vulgaris* primary leaves were electroporated, under the conditions indicated, with 25 μg of the *35S-CAT* fusion. Following transfection, protoplasts were incubated in 20 μmol m^{-2} s^{-1} white light for 20 h, harvested and CAT activity (cpm) determined.

(B) *Arabidopsis* leaf protoplasts were transfected with either 20 μg of either the *35S-GUS* or *CHS-GUS* fusion using PEG and transferred to darkness (D) or 50 μmol m^{-2} s^{-1} UV-A/blue light (B) for 20 h. Following incubation, protoplasts were harvested and assayed for GUS activity.

A

Protoplasts	Electroporation prameters	Relative CAT activity (cpm)
Phaseolus vulgaris leaf protoplasts	100 μF, 350 V	34,073
Arabidopsis leaf protoplasts	21 μF, 250 V	0
	21 μF, 500 V	0
	21 μF, 750 V	153
	21 μF, 1000 V	19
	21 μF, 1250 V	61
	100 μF, 100 V	252
	100 μF, 200 V	318
	100 μF, 300 V	12
	100 μF, 400 V	174
	100 μF, 500 V	0

B



CHAPTER 6: SIGNAL TRANSDUCTION REGULATING *PAL* GENE EXPRESSION

6.1 Introduction

The protein phosphatase inhibitor, cantharidin, stimulated *PAL* gene expression in *Arabidopsis* cells, but inhibited *CHS* gene expression. This implies that there are differences in the signal transduction pathways regulating *PAL* and *CHS*. The aim of the experiments described in this chapter was to investigate these differences. A pharmacological approach was used to identify likely signalling processes involved in the induction of *PAL* by cantharidin. The results demonstrate that cantharidin activates an early event in an, as yet unidentified, signalling pathway coupled to *PAL* gene expression. Moreover, our findings indicate that the cantharidin signalling pathway is different from the UV-B and UV-A/blue light signal transduction pathways regulating *PAL* and *CHS* in the *Arabidopsis* cell culture.

6.2 Cantharidin Induces a Rapid Accumulation of *PAL* Transcripts

Plants contain enzymes that are remarkably similar to three of the four classes of serine/threonine protein phosphatases found in animal cells, namely protein phosphatases 1, 2A and 2C (MacKintosh *et al.*, 1991). In both plants and animals, protein phosphatases 1 and 2A are inhibited by several toxins, including okadaic acid (Cohen *et al.*, 1990) and cantharidin (Li and Casida, 1992). Cantharidin is particularly useful as it is relatively inexpensive. Recently, MacKintosh *et al.* (1994) reported that a variety of phosphatase inhibitors, including okadaic acid and cantharidin, stimulate PAL activity when added to cultured soybean cells. Moreover, both phosphatase inhibitors induce the production of anti-fungal agents in soybean cotyledons, indicating a role for protein phosphorylation in plant defense against pathogen attack. Consistent with this, okadaic acid has been shown to induce pathogenesis-related transcript and

protein accumulation in tobacco leaves (Raz and Fluhr, 1993). Our previous results extend the observations of MacKintosh *et al.* (1994) and show that *PAL* transcipt levels increase following the addition of okadaic acid and cantharidin to the *Arabidopsis* cell culture (Fig. 4.8). As shown in Figure 6.1, incubation of cells with cantharidin causes a rapid increase in *PAL* transcripts which reaches a maximum after approximately 2 h. Transcripts deline sharply after 12 h exposure to cantharidin and by 24 h return to the basal level found in untreated cells. The rapid nature of this response suggests that *PAL* induction results from a direct increase in protein phosphorylation as opposed to more complex secondary effects. Okadaic acid has been shown to stimulate a rapid increase in protein phosphosphorylation in tobacco leaves (Raz and Fluhr, 1993). Thus protein phosphatase inhibitors may act to increase the phosphorylation state of a target protein(s) involved in a signalling pathway coupled to the expression of *PAL* and other plant defence genes. This possibility was investigated by examining the effects of specific agonists and inhibitors on the induction of *PAL* transcripts by cantharidin in the *Arabidopsis* cell culture.

6.3 Extracellular Calcium is Required for the Cantharidin Induction of *PAL*

The cantharidin response was previously used to determine whether the effects of signalling antagonists on the induction of *CHS* by UV/blue light, were specific or due to a general inhibition of gene expression. From these experiments, it was observed that the induction of *PAL* transcripts in response to cantharidin was severely attenuated in the presence of 10 mM EGTA (Fig. 4.9A). Similarly, Figure 6.2 shows that addition of 5 mM EGTA to *Arabidopsis* cells severely reduced *PAL* transcript accumulation in response to cantharidin. However, previous data indicated that EGTA may be having a spurious effect in these cells (see 4.10 and 4.11). Therefore, to determine whether calcium is involved in the cantharidin response, the effects of known calcium channel blockers were examined. Figure 6.2 shows that addition of the plasma

membrane calcium channel blocker, lanthanum, to the culture medium at 10 mM, inhibited the accumulation of *PAL* transcripts in response to cantharidin. Incubation of *Arabidopsis* cells with the voltage-operated calcium channel blocker, verapamil (100 μM), also inhibited *PAL* induction by cantharidin. The UV-B and UV-A/blue light induction of *CHS* expression was unaffected by both these compounds, demonstrating that their effects on *PAL* expression do not result from a general inhibition of transcription or acceleration of mRNA turnover (Fig. 4.1B). Therefore, these results indicate an involvement of calcium in the cantharidin signalling pathway regulating *PAL* in the *Arabidopsis* cell culture. Moreover, the inhibitory effect of lanthanum suggests an influx of calcium from the extracellular space.

6.4 An Increase in Cytosolic Calcium is Sufficient to Stimulate *PAL*

Since the above results indicated that an influx of calcium is involved for the cantharidin induction of *PAL*, it was investigated whether the artificial elevation of cytosolic calcium could stimulate *PAL* transcript accumulation. The calcium ionophore A23187, supplemented with 10 mM $CaCl_2$, was previously shown to increase cytosolic calcium in *Arabidopsis* cells, sufficient to induce *TCH3* gene expression (Fig. 4.2). Therefore, the effect of an ionophore/calcium treatment on *PAL* gene expression was examined. As shown in Figure 6.3, the ionophore/calcium treatment caused a rapid increase in *PAL* transcripts in the *Arabidopsis* cell culture reaching a maximum after approximately 2 h. Subsequently, *PAL* transcript levels decline but are still detectable after 12 h. These results therefore indicate that an increase in cytosolic calcium is sufficient to stimulate *PAL* expression in the *Arabidopsis* cell culture. Moreover, the inhibitor studies described above suggest that the artificial increase in cytosolic calcium may stimulate *PAL* transcript accumulation through the same signalling pathway that is activated by cantharidin. These observations are consistent with the hypothesis that protein phosphatase inhibitors increase the phosphorylation state of a particular cellular

component(s) which in turn activates a signalling pathway controlling *PAL* gene expression. It is possible that cantharidin directly stimulates an increase in cytosolic calcium from an extracellular pool.

6.5 Staurosporine and Cycloheximide Inhibit *PAL* Induction

In a simple biological system, a protein kinase inhibitor would be expected to oppose the effects of a protein phosphatase inhibitor. Thus if a phosphatase inhibitor activates a response, the reciprocal use of a kinase inhibitor may inhibit the response. Consistent with this, it was previously observed that the general serine/threonine protein kinase inhibitor, staurosporine, prevented *PAL* transcript accumulation in response to cantharidin (Fig. 4.9A). These results support the hypothesis that an increase in protein phosphorylation is responsible for the induction of *PAL* gene expression the *Arabidopsis* cell culture. If staurosporine simply acts to oppose the effects of cantharidin stimulating an increase in cytosolic calcium, then one would not expect it to affect the ionophore/calcium induction of *PAL* transcripts. However, Figure 6.4B shows that addition of staurosporine to the culture medium at 1 μM, inhibited *PAL* transcript accumulation in response to an increase in cytosolic calcium. Therefore, protein kinase activity appears to be required downstream of calcium in the cantharidin-activated signalling pathway regulating *PAL* in the *Arabidopsis* cell culture. However, from the above results, it is impossible to conclude whether staurosporine opposes the upstream effects of cantharidin in addition to blocking protein kinase activity downstream in the signal transduction pathway. More importantly, as staurosporine was previously shown to inhibit the induction of *CHS* and *TCH3* in response to UV/blue light and ionophore/calcium respectively, the possibility that it causes a general inhibition of gene expression in *Arabidopsis* cells cannot be excluded. Nevertheless, our findings are consistent with those of MacKintosh *et al.* (1994) who showed that K252a, at similar concentrations, blocks the induction of PAL activity by okadaic acid.

Similalrly, Raz and Fluhr (1993) observed that K252a inhibits pathogenesis-related transcript and protein accumulation in tobacco leaves in response to okadaic acid.

Mackintosh *et al.* (1994) also reported that the induction of PAL activity by okadaic acid is sensitive to inhibitors of cytoplasmic protein synthesis. We therefore investigated whether protein synthesis is required for the cantharidin induction of *PAL* in the *Arabidopsis* cell culture. As shown in Figure 6.4A, addition of cycloheximide to the cell culture abolishes the increase in *PAL* transcripts in response to cantharidin. The ionophore/calcium induction of *TCH3* is unaffected by cycloheximide, demonstrating the effect on *PAL* transcript accumulation is not due to a general inhibition of gene expression (Fig. 4.11B). To determine whether the requirement for protein synthesis is located upstream or downstream in the cantharidin-activated signalling pathway, the effect of cycloheximide on *PAL* induction by ionophore/calcium was examined. Figure 6.4B shows that incubation of cells with 20 μM cycloheximide inhibited *PAL* transcript accumulation in response to an increase in cytosolic calcium. Thus the requirement for protein synthesis is located downstream of calcium in the cantharidin-activated signalling pathway regulating *PAL* gene expression in the *Arabidopsis* cell culture. The fact that cycloheximide and staurosporine inhibit the induction of *PAL* by either canatharidin or ionophore/calcium is consistent with the conclusion that the same signal transduction pathway is being activated in each case.

6.6 UV-B and UV-A/blue Light Regulate *PAL* Transcript Accumulation in the *Arabidopsis* Cell Culture

PAL gene expression is induced by a number of environmental signals, including light (Hahlbrock and Scheel, 1989; Ohl *et al.*, 1990; Kubasek *et al.*, 1992). However, very little has been done to define the photoreceptors that mediate the effects of light on *PAL* expression in mature leaf tissue. It was therefore investigated whether *PAL* gene expression is regulated by UV-B and UV-A/blue light in the *Arabidopsis* cell culture. As shown in Figure 6.5, *PAL* transcripts show a large increase when *Arabidopsis* cells

are transferred to a 6-fold higher fluence rate of white light. Transfer of the culture to UV-B and UV-A/blue light also induces *PAL* transcript accumulation. However, no increase in *PAL* transcripts was observed in response to red light (data not shown). These findings demonstrate that the regulation of *PAL* gene expression by different light qualities in the *Arabidopsis* cell culture is very similar to that observed for *CHS* (Fig. 3.3C). Furthermore, they imply that *PAL* and *CHS* transcript accumulation are induced by the same UV/blue photoreceptor systems. To test this hypothesis, we examined the effects of specific antagonists on the UV-B and UV-A/blue light regulation of *PAL* gene expression in the *Arabidopsis* cell culture. Figure 6.6A shows that the calcium antagonists, EGTA, nifedipine and ruthenium red inhibit *PAL* transcript accumulation in response to UV-B and UV-A/blue light. In contrast, incubation of cells with lanthanum or verapamil has no significant inhibitory effect on *PAL* induction by UV-B and UV-A/blue light. Hence the effects of these compounds on *PAL* transcript accumulation are identical to those on *CHS* induction by UV-B and UV-A/blue light (Fig. 4.1). Similar inhibitory effects were observed with the calmodulin anatgonists, W-7, W-5 and trifluoperazine: addition of W-7 effectively inhibits the UV-B signalling pathway whereas trifluoperazine prevents transcript accumulation in response to both UV-B or UV-A/blue light (Fig. 6.6B). Although the UV-A/blue induction of *PAL* is weaker in this particular experiment, the effects of calmodulin antagonists on *PAL* transcript accumulation are similar to those observed for *CHS* (Fig. 4.3). Taken together, these results strongly suggest that *PAL* and *CHS* gene expression are controlled by identical UV-B and UV-A/blue phototransduction pathways in the *Arabidopsis* cell culture. Consistent with this conclusion is the observation that staurosporine and cycloheximide inhibit the UV-B and UV-A/blue light induction of *PAL* transcripts at concentrations shown to inhibit the induction of *CHS* transcripts (Fig. 6.7).

6.7 Cantharidin Enhances the UV-B and UV-A/blue Light Induction of *PAL* Transcripts

While okadaic acid and cantharidin induce *PAL* gene expression, we previously observed that these phosphatase inhibitors abolish the accumulation of *CHS* transcripts in response to UV-B and UV-A/blue light. The effect of cantharidin on the induction of *PAL* by UV-B and UV-A/blue light was therefore investigated. As shown in Figure 6.8, incubation of *Arabidopsis* cells with 200 μM cantharidin has no inhibitory effect on *PAL* transcript accumulation in response to both UV-B and UV-A/blue light. On the contrary, cantharidin appears to enhance the level of *PAL* transcripts in both light qualities. This suggests that cantharidin and UV/blue light mediate their effects on *PAL* gene expression through separate signalling pathways.

The pharmacological studies described above indicate a real difference between the cantharidin-activated signalling pathway and UV/blue phototransduction pathways regulating *PAL* in the *Arabidopsis* cell culture. However, the differential effect of cantharidin on the induction of *PAL* and *CHS* by UV/blue light requires further explanation. A possible interpretation is that the cantharidin-activated signalling pathway described here may somehow act to inhibit the light induction of *CHS* but stimulate *PAL* in the presence or absence of light. Interestingly, the latter hypothesis is very similar to the effects of light and fungal elicitor on *PAL* and *CHS* expression in cultured parsley cells (Lozoya *et al.*, 1991). Treatment of parsley cells with fungal elicitor completely represses the UV light induction of *CHS*, whereas *PAL* is induced by elicitor, UV light or both. The significance of the overlap in regulation between elicitor and UV light-mediated responses is thought to increase the flux through particular phenylpropanoid branch pathways concerned with the production of anti-fungal agents. *CHS* expression is not required for synthesis of these compounds in parlsey and is therefore repressed in the presence of elictor (Chappell and Hahlbrock, 1984; Lozoya *et al.*, 1991; Hahlbrock *et al.*, 1995). *CHS* expression in *Arabidopsis* cells is also not induced by fungal elicitors (Davis and Ausubel, 1989). Consistent with this, jasmonic acid, a mediator of pathogen attack, was previously shown to

stimulate *PAL* but not *CHS* in the *Arabidopsis* cell culture (Fig. 3.9). Thus a similar interaction between elicitor and light-mediated response pathways may occur in *Arabidopsis*. If so, our findings suggest that cantharidin may mediate its effects on *PAL* and *CHS* expression through a signalling pathway coupled to elicitor recognition. This is consistent with the observations that protein phosphatase inhibitors activate anti-fungal defense responses in soybean and tobacco (Raz and Fluhr, 1993; Mackintosh *et al.*, 1994). It is therefore a priority to determine whether fungal elicitors induce *PAL* transcript accumulation in the *Arabidopsis* cell culture system and to compare the features of this signalling pathway to those of the cantharidin-activated pathway.

6.8 Cantharidin and UV/blue Light do not Elicit a Rapid Increase in Cytosolic Calcium

Our results indicate a requirement for calcium in the cantharidin signalling pathway regulating *PAL* gene expression in the *Arabidopsis* cell culture. It is therefore possible that cantharidin stimulates an increase in cytosolic calcium. Several studies have used transgenic plants expressing a calcium-sensitive photoprotein of jellyfish, aequorin, to detect changes in cytosolic calcium in response to a variety of stimuli (e.g. Knight *et al.*, 1991, 1992; Ohto *et al.*, 1995; Haley *et al.*, 1995; Knight *et al.*, 1996). Thus this approach was used to examine whether cantharidin elicits an increase in cytosolic calcium. Cold shock, which induces a rapid increase in aequorin luminesence in transgenic tobacco plants (Knight *et al.*, 1991), was used as a control in our experiments. Transgenic *Arabidopsis* plants expressing cytosolic aequorin were grown in a low fluence rate of white light for 14 days and incubated in the dark for 24 h with coeleterazine to allow reconstitution of holoaequorin. As shown in Figure 6.9, low temperature elicits a large, immediate increase in cytosolic calcium levels in these plants. In contrast, no effect on aequorin luminescence was observed following immersion of plants in water equilibrated to room temperature (data not shown). While we were able to detect luminescence in response to cold shock, no significant increase

in cytosolic calcium was observed over a 5 min period after treating plants with various concentrations of cantharidin (100 μM up to 1 mM). However, it is possible that the failure of cantharidin to increase aequorin luminescence in transgenic *Arabidopsis* is due to a lack of uptake or that the increase has a slow kinetics.

Since the inhibitor studies implicated a role for calcium in the UV-B and UV-A/blue phototransduction pathways regulating *CHS* and *PAL* in the *Arabidopsis* cell culture, it was investigated whether these wavelengths could stimulate an increase in cytosolic calcium in transgenic plants. No rapid increase in aequorin luminescence was observed following a brief illumination with UV-B or UV-A/blue light (10 s up to 5 min) at fluence rates used to stimulate *PAL* and *CHS* expression (data not shown). The failure of UV-B and UV-A/blue light to induce an immediate increase in aequorin luminescence could be because the short illuminations used in these experiments were insufficient to activate the UV-B and UV-A/blue light phototransduction pathways regulating *CHS* and *PAL*. Alternatively, an increase in cytosolic calcium may only have been detected after a longer time scale (many minutes). Clearly, further research is required to investigate these possibilities.

6.9 Discussion

In the present chapter, we have used a pharmacological approach in the *Arabidopsis* cell culture to identify likely signalling processes concerned with the induction of *PAL* gene expression in response to cantharidin. Although some of the experiments presented in this chapter need to be repeated, the results indicate that cantharidin activates a signalling pathway which requires an influx of calcium, protein synthesis and protein kinase activity. Although not directly tested, we suggest that protein phosphatase inhibitors may activate *PAL* through an elicitor-mediated response pathway.

6.9.1 Similar UV-B and UV-A/blue phototransduction pathways appear to regulate *PAL* in the *Arabidopsis* cell culture

All phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine by the action of PAL (Fig. 1.1). This reaction represents the branch point between primary (shikimate pathway) and secondary (phenylpropanoid) metabolism in plants (Dixon and Piava, 1995). PAL is the first enzyme in a set of core reactions that provide precursors for a number of unique and important plant metabolites (Hahlbrock and Scheel, 1989). These include lignins, suberins, and a variety of phenolic esters that are found in plant cell walls. Other prominent phenylpropanoid products include the flavonoid derivatives which exhibit a wide range of biological functions (Koes *et al.*, 1994). Indeed, *PAL* gene expression, like *CHS*, is stimulated by a complex array of environmental and developmental signals (Hahlbrock and Scheel, 1989). In most plant species, *PAL* genes occur in small families of 2 to 6 members. For example, three *PAL* genes have been identified in *Arabidopsis* (Wanner *et al.*, 1995). *PAL1* and *PAL2* are structurally similar to *PAL* genes from other plant species whereas *PAL3* appears to be significantly different.

Studies have shown that *PAL* expression in *Arabidopsis* is induced by light (Ohl *et al.*, 1990; Kubasek *et al.*, 1992), fungal elicitors (Trezzini *et al.*, 1993), bacterial pathogens (Dong *et al.*, 1991; Leyva *et al.*, 1995), low temperature stress (Christie *et al.* 1994; Leyva *et al.*, 1995), cytokinins (Deikman and Hammer, 1995), wounding (Ohl *et al.*, 1990), jasmonic acid (Gundlach *et al.*, 1992) and ozone fumigation (Sharma and Davis, 1994). With respect to light, *PAL* gene expression has been studied extensively using the parsley cell culture system (Chappell and Hahlbrock, 1984; Lois *et al.*, 1989; Hahlbrock and Scheel, 1989; Hahlbrock *et al.*, 1995; Logemann *et al.*, 1995). However, the work on parsley has been carried out using a broadband white light source with a high blue and UV content. From these experiments, it is impossible to make definite statements concerning the photoreceptors involved.

In the *Arabidopsis* cell culture, *PAL* gene expression is regulated by UV-B and UV-A/blue light (Fig. 6.5), similar to the situation found in young *Arabidopsis* seedlings (Kubasek *et al.*, 1992). Moreover, the experiments reported here, in relation to our previous observations, indicate that *PAL* and *CHS* expression in the *Arabidopsis* cell culture are controlled by identical UV-B and UV-A/blue light signal transduction pathways (Fig. 6.6 and 6.7): both the UV-B and UV-A/blue phototransduction processes involve calcium. The UV-A/blue signalling pathway does not appear to involve calmodulin whereas the UV-B response does. Furthermore, both pathways appear to involve protein phosphorylation and require protein synthesis. The only difference is that protein phosphatase inhibitors inhibit the UV/blue light induction of *CHS* but not *PAL*. A possible reason for this discrepancy is discussed below.

Although our results indicate an involvement of calcium in the UV-B and UV-A/blue phototransduction pathways regulating *CHS* and *PAL* in the *Arabidopsis* cell culture, no rapid increase in aequorin luminescence was observed in transgenic *Arabidopsis* following a brief illumination with UV-B or UV-A/blue light. As a control, low temperature elicited a large, immediate increase in the same plants (Fig. 5.9). Nevertheless, these findings are consistent with the hypothesis developed in relation to the ionophore/calcium experiment in the previous chapter, that UV-B and UV-A/blue light trigger an increase in calcium that is not cytosolic (e.g. nuclear) or occurs in a particular subcellular microdomain (Fig. 4.2A). This hypothesis can now be investigated by using transgenic *Arabidopsis* containing aequorin targeted to particular subcellular locations (e.g. Knight *et al.*, 1996).

6.9.2 The cantharidin signalling pathway regulating *PAL*

Protein phosphatase activity is involved in regulating several enzymes of plant metabolism including PAL (Mackintosh *et al.*, 1991; MacKintosh *et al.*, 1994). Cantharidin, a specific inhibitor of animal and plant protein phosphatases 1 and 2A (Honkanen, 1993) is shown here to stimulate a rapid increase in *PAL* transcripts in the

Arabidopsis cell culture (Fig. 6.1). From pharmacological studies with a range of well established signalling antagonists, some of the processes involved in the cantharidin signal transduction pathway regulating *PAL* expression in the *Arabidopsis* cell culture have been identified. The location of the inhibitior-sensitive components within the cantharidin signalling pathway can be mapped and their positions, based on the current information, are shown in Figure 6.10. Our observations indicate that cantharidin functions upstream in an, as yet unidentified, response pathway coupled to the induction of *PAL* gene expression. Cantharidin presumably mediates its effect on *PAL* by increasing the phosphorylation state of a particular cellular target protein(s). However, it is not known which type of kinase activity is involved in phosphorylating the target(s) of cantharidin action. This in turn, leads to an increase in cytosolic calcium which can be inhibited by lanthanum or verapamil (Fig. 6.2). The effects of these compounds suggest that external calcium is required and that calcium uptake involves a verapamil-sensitive plasma membrane calcium channel. Support for the involvement of extracellular calcium comes from the observation that ruthenium red, an inhibitor of mitochondrial and endoplasmic reticulum calcium channels (Knight *et al.*, 1992; Allen *et al.*, 1995; Monroy and Dhindsa *et al.*, 1995), has no effect on the cantharidin response (Fig. 4.9A). Moreover, this event can be mimicked by addition of ionophore/calcium to the cell culture medium (Fig 6.3). Despite this, cantharidin failed to elicit a rapid, increase calcium in transgenic *Arabidopsis* expressing cytosolic aequorin. However, while protein phosphatase inhibitors are taken up freely into excised leaves through the petiole (MacKintosh *et al.*, 1994), they may be less able to permeate whole plants. Further experiments using excised leaves or, more ideally, transgenic cells expressing aequorin, are required to determine whether an increase in cytosolic calcium occurs in response to cantharidin treatment.

The inhibitory effects of cycloheximide and staurosporine indicate that the cantharidin signalling pathway subsequently involves protein synthesis and the activity of a protein kinase(s) (Fig. 6.4). However, from the current information, it is impossible to make any conclusions regarding their downstream positions in the

pathway. Hence these events are placed together in the proposed model. It is likely that a component(s), such as a transcription factor, is synthesised which is essential for the activation of gene expression. Indeed, a downstream staurosporine-sensitive protein kinase might regulate the activity of such a component(s). Protein factors that may be important in regulating *PAL* gene expression have recently been reported (Hahlbrock *et al.*, 1995). It will be interesting to see whether the expression of these protein factors is induced by cantharidin and other protein phosphatase inhibitors.

The results also indicate that the cantharidin signalling pathway is distinct to the UV/blue phototransduction pathways regulating *PAL* in the *Arabidopsis* cell culture. Firstly, these pathways appear to have different calcium requirements: the cantharidin pathway requires an influx of calcium and is sensitive to lanthanum and verapamil, whereas the UV/blue phototransduction pathways appear to involve an internal source of calcium and are sensitive to nifedipine and ruthenium red. Secondly, in contrast to the UV-B and/or UV-A/blue light signalling processes, W-7 and trifluoperazine have no inhibitory effect on the cantharidin induction of *PAL*, indicating that calmodulin is not required for this response (Fig. 4.9A). Finally, cantharidin appears to enhance both the UV-B and UV-A/blue light induction of *PAL* transcripts (Fig. 6.8). Whether this is due to an additive effect of both stimuli or a greater accumulation of *PAL* transcripts in response to cantharidin relative to UV/blue light remains to be established.

6.9.3 Cantharidin may activate an elicitor-mediated signalling pathway regulating *PAL*

In response to pathogen attack, plants accumulate a diverse range of compounds which act as anti-microbial agents. These so-called phytoalexins can also be induced by elicitors derived from fungal cell walls or culture filtrates (Dixon *et al.*, 1994; Hahlbrock *et al.*, 1995). Phytoalexins are derived from general phenylpropanoid metabolism which converts phenylalanine to 4-coumaroyl-CoA (Fig. 1.1). Transcription of the genes encoding the enzymes of general phenylpropanoid

metabolism, PAL, cinnamate 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL), is induced by elicitor treatment in parsley cell cultures (Chappell and Hahlbrock, 1984; Logemann *et al.*, 1995). Recent evidence suggests that protein phosphatase inhibitors mimic the elicitor-induced defense response. Firstly, okadaic acid has been reported to induce PAL activity and isoflavanoid phytoalexin production in soybean (MacKintosh *et al.*, 1994). Indeed, our results imply that PAL induction by protein phosphatase inhibitors results from an increase in *PAL* transcript accumulation. Secondly, addition of okadaic acid to soybean cells elicits a rapid alkalinisation of the culture medium (MacKintosh *et al.*, 1994), a common response of plant cells to fungal elicitors (Dixon *et al.*, 1994) which presumably results from the regulation of specific ion channels at the plasma membrane (Mathieu *et al.*, 1994). More recently, the phosphatase inhibitor, calyculin A, has been reported to behave like an elicitor-mimicking molecule and trigger extracellular alkalinisation when added to tobacco cells (Mathieu *et al.*, 1996). Protein phosphatase inhibitors may therefore activate an early event in the signal transduction pathway coupling elicitor recognition to *PAL* activation and hence phytoalexin production. Consistent with this hypothesis, rapid changes in protein phosphorylation have been observed in response to fungal elicitors in parsley and tomato cell cultures (Dietrich *et al.*, 1990; Felix *et al.*, 1991). Moreover, evidence presented here indicates that cantharidin acts upstream in a signalling pathway requiring an influx of calcium, protein synthesis and protein kinase activity. Interestingly, these processes are associated with elicitor-mediated signal transduction in several plant systems (Grosskopf *et al.*, 1990; Knight *et al.*, 1991; Messiaen *et al.*, 1993; MacKintosh *et al.*, 1994; Nürnberger *et al.*, 1994; Susuki *et al.*, 1995).

In parsley, there appears to be an overlap in the regulation of elicitor and UV light-mediated responses (Hahlbrock *et al.*, 1995). While the enzymes of general phenylpropanoid metabolism, PAL, 4CH and 4CL, are strongly induced by both fungal elicitor and UV light (Chappell and Hahlbrock, 1984; Logemann *et al.*, 1995), subsequent phenylpropanoid branch pathways are regulated in a more stimulus-specific manner. For instance, induction of CHS, the first committed enzyme of the flavonoid

branch pathway, is strongly dependent on specific light qualities (Ohl *et al.*, 1989; Frohnmeyer *et al.*, 1992) but not elicitor (Chappell and Hahlbrock, 1984). A simultaneous treatment of parsley cells with fungal elicitor and UV light prevents the light-stimulated increase in *CHS* expression, whereas *PAL* is induced by light, elicitor or both (Lozoya *et al.*, 1991). Although unexpected, a similar overlap in the regulation of *PAL* and *CHS* gene expression was observed in response to cantharidin and UV/blue light in the *Arabidopsis* cell culture. Cantharidin inhibits the UV/blue light induction of *CHS* whereas *PAL* gene expression is strongly induced by either cantharidin or UV/blue light. These findings therefore provide additional support that cantharidin may activate an elicitor-mediated signal transduction pathway regulating *PAL*. It will be important to determine whether fungal elicitors mediate similar effects on *PAL* and *CHS* expression in the *Arabidopsis* cell culture. Interestingly, elicitor treatment has been shown to repress light-stimulated *rbcS* transcription in potato leaves (Kombrink and Hahlbrock, 1990). More recently, Sheen (1993) reported that the light induction of *rbcS* expression in maize is stongly inhibited by protein phosphatase inhibitors. Thus the elicitor/phosphatase repression mechanism described here may also be applicable to other light-regulated genes.

Figure 6.1. Cantharidin induces a rapid increase in *PAL* transcripts

Cells grown in 20 μmol m^{-2} s^{-1} white light were treated with 200 μM cantharidin and incubated in 20 μmol m^{-2} s^{-1} white light for the times indicated. Equal amounts of RNA (20 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

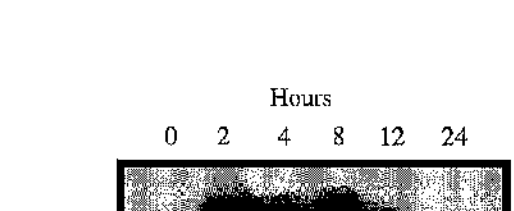

Hours
0 2 4 8 12 24
PAL
H1

Figure 6.2. Effects of calcium antagonists on the cantharidin regulation of *PAL* expression

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with increasing concentrations of EGTA (mM), 10 mM lanthanum (LA) or 100 μM verapamil (VER), prior to the addition of 200 μM cantharidin. Cells were harvested for RNA extraction after 6 h exposure to cantharidin. Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

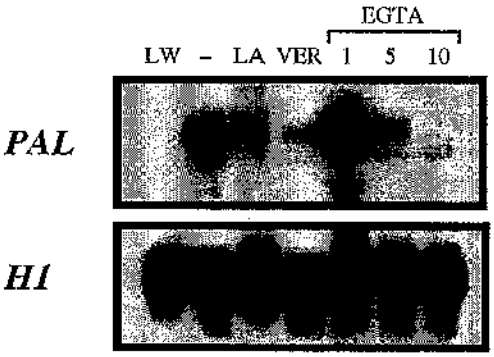

EGTA
LW – LA VER 1 5 10
PAL
H1

Figure 6.3. Elevating cytosolic calcium increases *PAL* transcript levels

Cells grown in 20 μmol m^{-2} s^{-1} white light were treated with 10 μM A23187 and 10 mM $CaCl_2$ and incubated in 20 μmol m^{-2} s^{-1} white light for the times indicated. Equal amounts of RNA (20 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

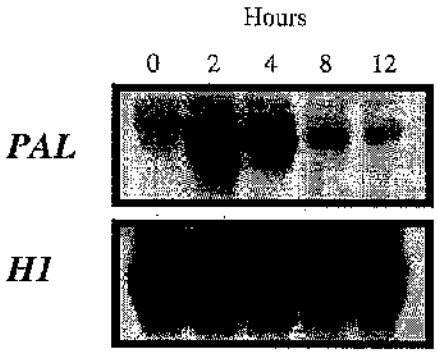
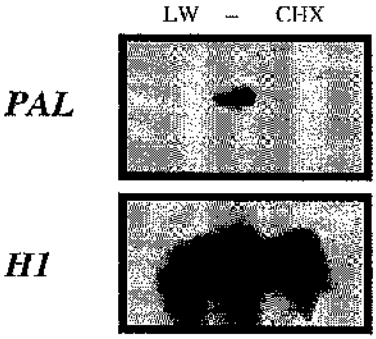

Hours
0
2
4
8
12
PAL
H1

Figure 6.4. Effects of staurosporine and cycloheximide on *PAL* gene expression

(A) Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with 20 μM cycloheximide (CHX) prior to the addition of 200 μM cantharidin. Cells were harvested after 6 h and total RNA isolated. Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

(B) Cells were grown and incubated either without (–) or with 20 μM cycloheximide (CHX) or 1 μM staurosporine (ST) as in (A). 10 μM A23187 and 10 mM $CaCl_2$ were then added and the cells incubated in 20 μmol m^{-2} s^{-1}. Cells were harvested after 4 h and total RNA isolated. LW6 represents cells incubated in low white light for a further 4 h without an ionophore/calcium treatment. A northern blot of total RNA (20 μg per lane) was hybridised to the *Arabidopsis PAL* probe and rehybridised to the *Phaseolus vulgaris H1* probe.

A



B

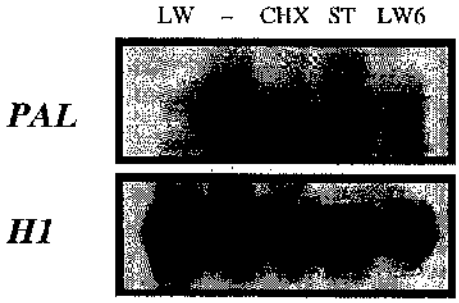


Figure 6.5. UV-B and UV-A/blue light induce *PAL* transcript accumulation

Cells grown in 20 μmol m^{-2} s^{-1} white light were transferred to the following light qualities for 6 h: 20 μmol m^{-2} s^{-1} white (lane 1), 120 μmol m^{-2} s^{-1} white (lane 2), 3 μmol m^{-2} s^{-1} UV-B (lane 3) or 80 μmol m^{-2} s^{-1} UV-A/blue (lane 4). RNA was isolated, 20 μg separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis PAL* probe, washed and autoradiographed. The membrane was then stripped of radioactivity and rehybridised to a *Phaseolus vulgaris H1* probe.

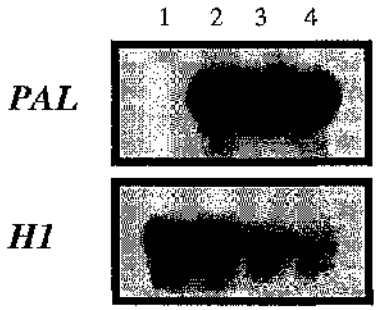
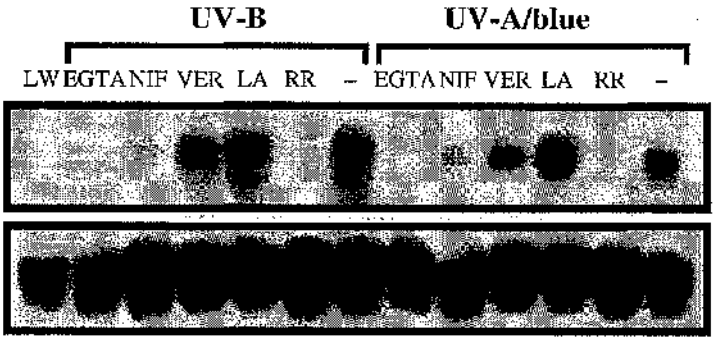

1 2 3 4
PAL
H1

Figure 6.6. Effects of calcium/calmodulin antagonists on the UV-B and UV-A/blue light regulation of *PAL* gene expression

(A) Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with 15 mM EGTA, 50 μM nifedipine (NIF), 100 μM verapamil (μM) or 50 μM ruthenium red (RR) prior to illumination with UV-B (3 μmol m^{-2} s^{-1}) or UV-A/blue light (80 μmol m^{-2} s^{-1}). Cells were harvested for RNA extraction after 6 h exposure to UV-B or UV-A/blue light. Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

(B) Cells grown as in (A) were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with increasing concentrations (μM) of W-7, W-5 or 25 μM trifluoperazine (TFP) prior to illumination as in (A). Transcripts were analysed as in (A).

A


UV-B
UV-A/blue
LW EGTA NIF VER LA RR – EGTA NIF VER LA RR –
PAL
H1

B

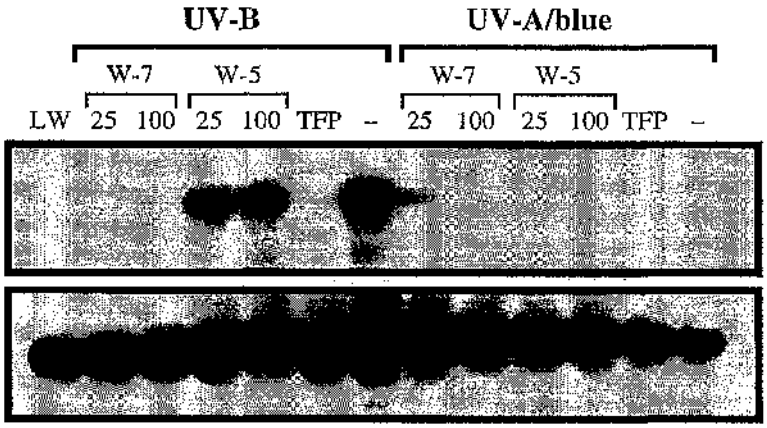
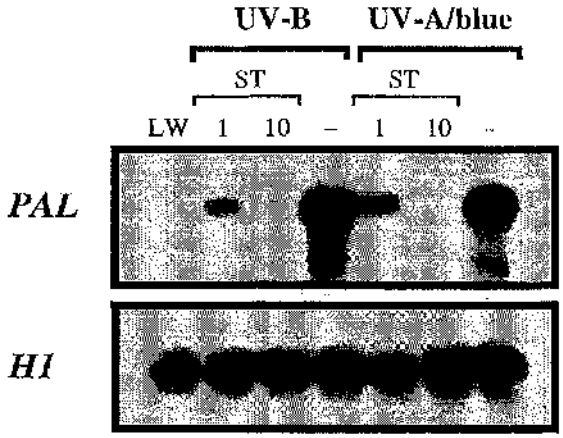

UV-B
UV-A/blue
W-7
W-5
W-7
W-5
LW 25 100 25 100 TFP – 25 100 25 100 TFP –
PAL
H1

Figure 6.7. Effects of staurosporine and cycloheximide on the UV-B and UV-A/blue light induction of *PAL* transcripts

(A) Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with 1 or 10 μM staurosporine (ST) prior to illumination for 6 h with UV-B (3 μmol m^{-2} s^{-1}) or UV-A/blue light (80 μmol m^{-2} s^{-1}). Equal amounts of RNA (10 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

(B) Cells grown as in (A) were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with 20 μM cycloheximide (CHX) prior to illumination as in (A). Transcripts were analysed as in (A).

A


UV-B
UV-A/blue
ST
ST
LW 1 10 – 1 10 –
PAL
H1

B

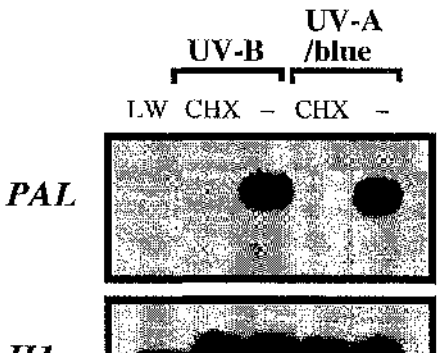

UV-A
/blue
UV-B
LW CHX – CHX –
PAL
H1

Figure 6.8. Cantharidin enhances the UV-B and UV-A/blue light induction of *PAL* transcript accumulation

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated for 1 h in 20 μmol m^{-2} s^{-1} white light (LW) either without (–) or with 200 μM cantharidin (CAN) prior to illumination for 6 h with UV-B (3 μmol m^{-2} s^{-1}) or UV-A/blue light (80 μmol m^{-2} s^{-1}). Equal amounts of RNA (20 μg) were separated on a 1.3% denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

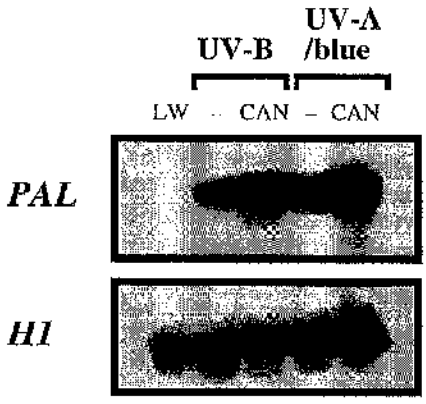

UV-A
/blue
UV-B
LW
CAN
CAN
PAL
H1

Figure 6.9. Cold-induced calcium-dependent aequorin luminescence in transgenic *Arabidopsis* plants

Trangenic *Arabidopsis* plants expressing cytosolic aequorin were grown in 20 μmol m^{-2} s^{-1} white light for 2 weeks. A single plant pre-incubated in 2 μM coelenterazine, was placed into a 3 ml plastic cuvette and transferred to a luminescence spectrophotometer. Using a 3 ml syringe, 2 ml ice-cold dH_2O was added to the cuvette at the time indicated by the arrow and luminescence monitored. Addition of dH_2O, equilibrated to room temperature, had no effect on aequorin luminescence.

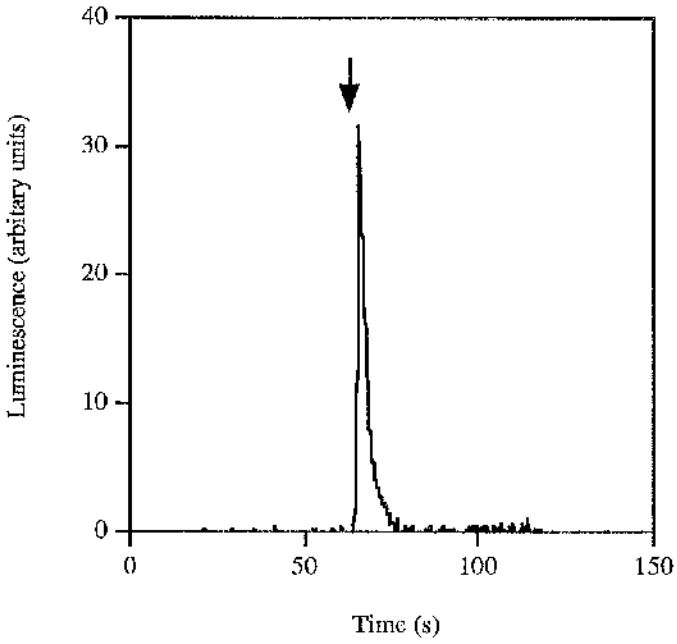
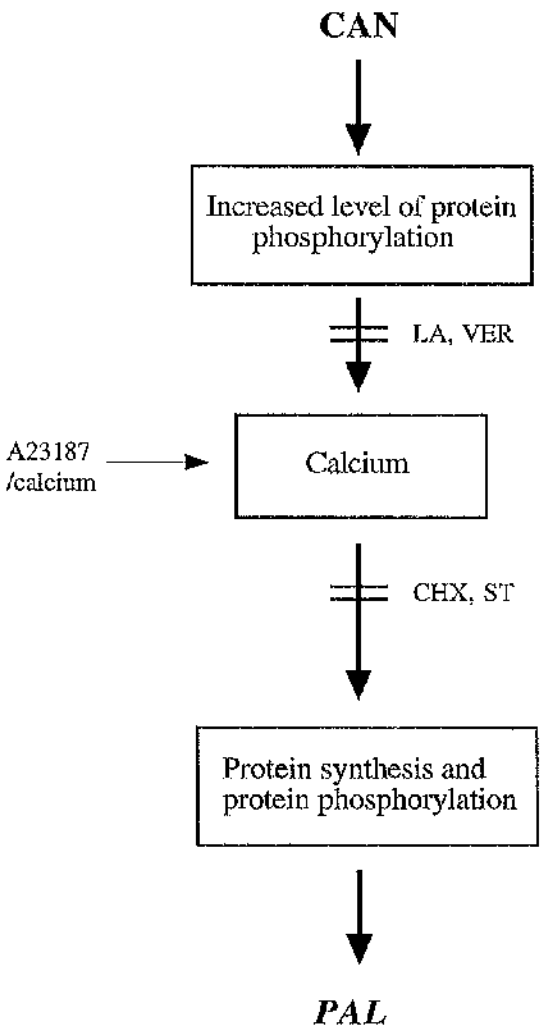

40
30
20
10
0
Luminescence (arbitary units)
0
50
100
150
Time (s)

Figure 6.10. Proposed model summarising the cantharidin signalling pathway regulating *PAL* gene expression

Pharmacological studies with the *Arabidopsis* cell culture indicate that cantharidin acts to regulate an early event in a, as yet unidentified, response pathway. As illustrated, cantharidin may activate this pathway by increasing the phosphorylation state of a particular cellular target(s). Subsequent signal transduction events appear to involve an increase in intracellular calcium, protein phosphorylation and protein synthesis. The positions of inhibitor sensitive components are indicated by two parallel lines drawn across the arrows in the pathway: lanthanum (LA), verapamil (VER), stauroporine (ST) and cycloheximide (CHX).


CAN
Increased level of protein phosphorylation
LA, VER
A23187 /calcium
Calcium
CHX, ST
Protein synthesis and protein phosphorylation
PAL

CHAPTER 7: FINAL DISCUSSION

7.1 Introduction

Pharmacological experiments in cultured cells or protoplasts provide a very useful approach to obtain information on the signal transduction processes involved in the regulation of plant gene expression. The work presented in this study demonstrates that a dissection of the signal response pathways controlling the expression of the flavonoid biosynthesis genes, *CHS* and *PAL*, is experimentally feasible with the *Arabidopsis* cell culture system. The aim of this chapter is to discuss and draw together the main conclusions of this research and summarise the prospects for future work.

7.2 Cultured Cells of *Arabidopsis* Permit Biochemical Analysis of UV-B and UV-A/blue Light Signal Transduction

In this study a photomixotrophic *Arabidopsis* cell suspension culture was used to study UV/blue light signal transduction. These cells show the same responses to different light qualities in the regulation of *CHS* expression as mature *Arabidopsis* plants (Fig. 3.3). That is, *CHS* transcript levels are induced by UV-B and UV-A/blue light rather than red and far-red light (Fig. 3.3C). Thus, in contrast to other species, such as soybean and tomato (Neuhaus *et al.*, 1993, Bowler *et al.*, 1994a, 1994b), there appears to be little or no induction mediated by phytochrome. Experiments with the newly established *Arabidopsis hy4* cell culture provide genetic evidence that separate UV-B and UV-A/blue light detection systems regulate *CHS* expression, the latter involving CRY1 (Fig. 3.9). This is consistent with the regulation pattern found in mature leaf tissue (G. Fuglevand, J.A. Jackson and G.I. Jenkins, unpublished). Following exposure to UV-B and UV-A/blue light *CHS* transcript levels increase within a few hours, which facilitates biochemical studies of the signal transduction processes (Fig. 3.5).

The light-induced expression of *CHS* and other genes has been studied in a number of cell systems, including parsley and soybean (Bowler *et al.*, 1994b; Hahlbrock *et al.*, 1995). In parsley and *Arabidopsis* leaves, *CHS* expression takes place in the epidermis (Schmelzer et. al., 1988; Chory and Peto, 1990). Thus cultured cells appear to display the characteristics of an epidermal cell line, providing a system of reduced complexity in which to study *CHS* gene expression. However, it seems improbable that each cell suspension culture originated from epidermal or vascular tissue. Indeed, the *Arabidopsis* cell culture described here is green, indicating the presence of chloroplasts, a feature not found in non-photosynthetic, epidermal cells. It is therefore likely that cultured cells have lost some developmental factor which acts to suppress *CHS* expression in leaf cell types other than epidermal and vascular tissues. Such developmental differences may also account for the lack of synergistic interaction between UV-B, UV-A and blue light on *CHS* expression in the *Arabidopsis* cell culture (Fig. 3.6C). That is, the CRY1-independent UV-A and blue light 'synergism' pathways identified in mature leaf tissue (G. Fuglevand, J.A. Jackson and G.I. Jenkins, unpublished) may be associated with a particular mode of cell type-specific gene expression.

7.3 UV-B and UV-A/blue Light Perception

While the induction of *CHS* expression by UV-A/blue light in *Arabidopsis* leaves and cultured cells is mediated by CRY1, the mechanisms involved in UV-B photoreception are still poorly understood. One possibility is that DNA itself acts as a UV-B detection system: UV-B-induced formation of pyrimidine dimers may result in the formation of some kind of 'signal' which stimulates the transcription of specific genes. This type of damage can generally be repaired by a simultaneous or subsequent irradiation with UV-A/blue light or white light including these wavelengths, in a process known as photoreactivation (Batschauer, 1993; Sancar, 1994). Indeed, some UV-induced plant responses are reversed by irradiation with UV-A/blue or white light, including

isoflavonoid production in *Phaseolus vulgaris* primary leaves (Beggs *et al.* 1985). In constrast, the UV-B induction of *CHS* is not reversible by UV-A/blue light in both *Arabidopsis* plants and cells (Fig. 3.6). This suggests that the accumulation of *CHS* transcrips in response to UV-B irradiation is not mediated by a photoreactivated type of DNA damage.

A more attractive hypothesis is that UV-B wavelengths are detected by a photoreceptor molecule analogous to other photoreception systems in higher plants. Although no information is available regarding the likely nature of the UV-B photoreceptor apoprotein, the absorption spectra of pterins make them suitable candidates for chromophore pigments (Galland and Senger, 1988b; Schmidt *et al.*, 1990). In addition, experiments with compounds which interact with flavins indicate that UV-B photoreceptors are likely to be proteins with a bound flavin chromophore (Ensminger and Schäfer, 1992; Khare and Guruprasad, 1993). However, from our results it appears that some of these compounds are toxic to plant cells, thus questioning their specificity as flavin antagonists.

A further mechanism of UV-B induction of gene expression may be the ability of UV-B to generate reactive oxygen species (ROS), such as a singlet oxygen. In support of this hypothesis, Green and Fluhr (1995) have recently reported that ROS mediate the expression of a pathogenesis-related protein, PR-1, in response to UV-B in tobacco leaves. This UV-B response was strongly attenuated in the presence of antioxidants. Moreover, rose bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) an inducer of ROS, was able to substitute for UV-B irradiation in inducing PR-1 accumulation. It would be of interest to determine whether there is a ROS involvement in the UV-B signalling pathway regulating *CHS*. Preliminary experiments with the *Arabidopsis* cell culture suggest that the UV-B induction of *CHS* does not involve ROS (J.C. Long and G.I. Jenkins, unpublished).

Clearly, further studies are required to improve our understanding of the UV-B detection system responsible for stimulating *CHS* expression. The isolation of mutants specifically altered in the UV-B induction of *CHS* will facilitate the identification of the

putative receptor and enable cloning of the corresponding gene(s). Transgene expression screens (Jackson *et al.*, 1995; Jenkins *et al.*, 1995) have already been undertaken in UV-B to identify *Arabidopsis* mutants with altered *CHS* expression (G. Fuglevand and G.I. Jenkins, unpublished). This has resulted in the isolation of mutants with increased or decreased expression of *CHS* transcripts in UV-B. Further research will establish whether these mutants are altered in UV-B photoreception *per se*.

7.4 UV-B and UV-A/blue Light Signal Transduction

We have identified likely signalling components of the UV-B and UV-A/blue phototransduction pathways regulating *CHS* expression in *Arabidopsis* cells. In contrast to the phytochrome signal transduction pathway regulating *CHS* in tomato and soybean (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994, 1994b), the signalling pathways mediating the UV-B and UV-A/blue light induction of *CHS* appear to involve cellular calcium because they are inhibited by the calcium channel blockers, nifedipine and ruthenium red (Fig. 4.1). Furthermore, there is no induction by cGMP and other mediators of the phytochrome signalling pathway (Fig. 4.12). The failure of lanthanum to inhibit the UV-B and UV-A/blue light induction of *CHS* suggests that an intracellular pool of calcium may be involved in the UV-B and UV-A/blue phototransduction. The UV-B and UV-A/blue pathways are also unaffected by the phenylalkylamine class of voltage-operated calcium channel blocker, verapamil. However, both lanthanum and verapamil stongly inhibit the canatharidin induction of *PAL*, demonstrating that these compounds are effective in our system (Fig. 6.2). The artificial elevation of cytosolic calcium using an ionophore and a channel agonist was insufficient on its own to stimulate *CHS* expression (Fig. 4.2), suggesting either that an additional process is required in conjunction with the increase in calcium, or a general increase in cytosolic calcium is not involved. Consistent with the latter hypothesis, in preliminary experiments with transgenic *Arabidopsis* containing

cytosolic aequorin (Knight *et al.*, 1991) we have not observed any significant, rapid increase in calcium in response to UV-B or UV-A/blue light. Further experiments, using transgenic *Arabidopsis* containing aequorin targeted to specific subcellular locations (Knight *et al.*, 1996), are required to examine whether UV-B and UV-A/blue light elicit an increase in calcium in a particular microdomain or organelle.

Both the UV-B and UV-A/blue light induction of *CHS* are likely to involve protein phosphorylation since the responses are inhibited by protein kinase and phosphatase inhibitors (Fig. 4.6 and 4.7). Evidence was also obtained that *CHS* induction by UV-B and UV-A/blue light requires protein synthesis, since cycloheximide inhibited these responses (Fig. 4.11). In this study, the only difference detected between the UV-B and UV-A/blue phototransduction pathways is that calmodulin appears to be involved in the UV-B pathway but not the UV-A/blue pathway. The evidence for this is that *CHS* expression in response to UV-B is strongly inhibited by the the calmodulin antagonist W-7, whereas the UV-A/blue induction is hardly affected (Fig. 4.3). This provides strong evidence that the UV-B and UV-A/blue phototransduction pathways are, at least in part, distinct. The inhibitory effect of trifluoperazine on the UV-A/blue light induction of *CHS* suggests that another type of calcium-binding protein may be involved in this response.

In *Arabidopsis* cells, UV-B and UV-A/blue light were also found to elevate *PAL* transcript levels (Fig. 6.5), similar to the situation found in whole plants (Kubasek *et al.*, 1992). However, studies of signal transduction coupled to *PAL* gene expression have primarily focussed on induction by pathogen attack (Dixon *et al.*, 1994; Hahlbrock *et al.*, 1995). Pharmacological experiments with the *Arabidopsis* cell culture indicate that *PAL* transcript levels are induced by the same UV-B and UV-A/blue phototransduction pathways regulating *CHS* expression. This evidence is based on the similarities between the effects of specific antagonists on the UV-B and UV-A/blue light-induced accumulation of *PAL* and *CHS* transcripts (Fig. 6.6 and 6.7). It is now a priority to establish whether the *Arabidopsis hy4* mutant is impaired in the UV-A/blue, but not the UV-B, light induction of *PAL* gene expression.

The above experiments therefore give information on the cellular processes involved specifically in the UV-B and UV-A/blue phototransduction pathways inducing the transcription of genes required for flavonoid biosynthesis. For these studies, the induction of *PAL* transcripts by cantharidin or the induction of *TCH3* transcipts by ionophore/calcium was used as a control to show that compounds were not generally inhibiting gene expression or affecting cell viability. However, some reagents, at the concentrations tested, appeared to cause a general inhibition of transcriptional activation in *Arabidopsis* cells or protoplasts (see 4.10, 4.11 and 5.9). This demonstrates that caution should be exercised when interpretating such pharmacological data in the absence of appropriate controls. The fact that *H1* transcript levels were unaffected by any of the compounds used in this study indicates that consitutively expressed transcripts may not be ideal controls for assessing whether particular inhibitors are generally detrimental to gene expression.

Although the pharmacological data described above provide new information on the signal transduction pathways regulating gene expression in response to UV-B and UV-A/blue light, it is now important to demonstrate directly the activities inferred from the inhibitor studies. For instance, further experiments with transgenic *Arabidopsis* plants or cells expressing aequorin are required to establish whether UV/blue light can trigger an increase in calcium which occurs in a particular microdomain or organelle. Polisensky and Braam (1996) have recently demonstrated the feasibilty of generating transgenic *Arabidopsis* cell cultures expressing cytosolic aequorin to study intracellular calcium increases in response to low temperature. Additional experiments are also needed to investigate the nature of the serine/threonine kinase activity required for the UV/blue light induction of *CHS* and *PAL* in the *Arabidopsis* cell culture. A daunting task of future research will be to understand how the UV/blue light signal transduction processes interact with the underlying complexity of the COP-DET-FUS class of master regulators in the light signalling network.

7.5 Coupling UV-B and UV-A/blue Light Signal Transduction to Gene Expression

Many photomorphogenic responses require changes in both nuclear and chloroplast gene expression. Hence the binding of transcription factors to *cis*-regulatory promoter sequences is considered to be the terminal step of the signal transduction pathway. The transient expression system developed here offers a rapid means of analysing essential *cis*-acting sequences required for the UV/blue light induction of transcription of flavonoid biosynthesis genes. This system works well for protoplasts isolated from either the *Arabidopsis* cell culture (Fig. 5.2B) or mature *Arabidopsis* leaf tissue (Fig. 5.15). However, the leaf protoplast system may not be suitable for studying *CHS* or *PAL* promoter function since the light stimulation of these genes is restricted to the epidermal layer (Schmelzer *et al.*, 1988; Chory and Peto, 1990; Hahlbrock *et al.*, 1995). Cell culture protoplasts, on the other hand, show the same responses to different light qualities in the induction of *CHS* transcripts (Fig. 5.1B) and *CHS* promoter activity (Fig. 5.6) as *Arabidopsis* cells. Thus transient expression assays of *CHS-GUS* promoter-reporter fusions enabled the functional elements of the *Arabidopsis CHS* promoter to be dissected. The *Arabidopsis* promoter region contains a sequence with homology to the LRU1 of the parsley *CHS* gene, which mediates UV light regulation in parsley protoplasts (Weisshaar *et al.*, 1991a). Our studies reveal that the *Arabidopsis CHS* LRU is both necessary and sufficient for the induction of transcription specifically by UV-B, as well as UV-A/blue light (Fig 5.9 and Fig. 5.10). We therefore conclude that the UV-B and UV-A/blue phototransduction pathways terminate with an effect on the transcription factors binding to the LRU region. Similarly, Merkle *et al.* (1994) reported that UV-B and blue light produce the same in vivo footprint pattern in the parsley *CHS* promoter. Frohnmeyer *et al.* (1994) showed that even evacuolated parsley protoplasts retain light-responsiveness, indicating that the photoreceptors and signal transduction components involved are not located at the tonoplast or within the vacuole.

From the pharmacological experiments described above, it is likely that UV-B and UV-A/blue light regulate the synthesis and/or the activation of relevant transcription factors that bind to the LRU. Indeed, evidence suggests that both types of regulation may be involved. Transcripts encoding the parsley CPRF1, which interacts with the box II sequence element of LRU1, are rapidly induced by UV-containing white light prior to *CHS* transcript accumulation (Weisshaar *et al.*, 1991b; Feldbrügge *et al.*, 1994). There is also evidence that transcription factors which interact with box II are regulated by phosphorylation. Harter *et al.* (1994) reported that transcription factors which interact with box II are present in the cytosol and become activated and transported to the nucleus following illumination in reactions that involve protein phosphorylation. Whether UV-B and UV-A/blue light regulate the same transcription factors remains to be established. It is entirely possible that the UV-B and UV-A/blue phototransduction pathways merge upstream from *CHS* promoter interaction. However, the possibility that different transcription factors mediate the effects of UV-B and UV-A/blue light by binding to the *Arabidopsis CHS* LRU cannot be dismissed.

Information is also available regarding the DNA sequence elements present in the promoters of the parsley *PAL* genes which are concerned with UV light regulation (Hahlbrock *et al.*, 1995). The situation is complex as there appears to be an interaction between pathogen and UV light-triggered responses inducing *PAL* gene expression. This is reflected in the partial overlap of *cis*-acting elements involved in the fungal elicitor and UV light-mediated transcription of the *PAL-1* gene, identified by *in vivo* DNA footprinting (Lois *et al.*, 1989): boxes P, A and L are required for elicitor-responsiveness whereas boxes P and L are required for UV light-responsiveness. Indeed, box L shows strong sequence homology to box I of the parsley *CHS* promoter (Lois *et al.*, 1989). However, transient expression studies have demonstrated that neither box P, A, or L, or the promoter region containing them all together, confer elicitor or UV light-responsiveness on a GUS reporter gene (Logemann *et al.*, 1995). These elements are therefore necessary but not sufficient for elicitor or light-mediated transcription. Thus, the molecular mechanisms regulating the transcription of *PAL* by

UV-B and UV-A/blue light appear to involve a considerable degree of complexity. The three members of the *Arabidopsis PAL* gene family have recently been cloned (Wanner *et al.*, 1995). Two of these genes (*PAL1* and *PAL2*) are structurally similar to *PAL* genes isolated form other plant species, including parsley. The cell culture protoplast system described here will be a useful tool in establishing whether a similar interaction between elicitor and light-mediated responses occurs in *Arabidopsis*.

7.6 Protein Phosphorylation Appears to be Involved in the Regulation of *CHS* and *PAL*

While fungal elicitors induce a large number of genes in parsley cells, they have also been reported to down-regulate the expression of several other genes, including *CHS* (Hahlbrock *et al.*, 1995). The significance of this is that it is thought to increase the flux through particular phenylpropanoid branch pathways concerned with the production of anti-fungal agents (so-called phytoalexins). Thus PAL, the first enzyme of the phenylpropanoid pathway, is induced by either light or elicitor whereas CHS, the key point which commits the pathway to flavonoid biosynthesis is induced by light but is repressed in the presence of fungal elicitor (Lozoya *et al.*, 1991; Hahlbrock *et al.*, 1995). Transient expression studies have revealed that the parsley *CHS* LRU1 is necessary for negative elicitor-responsiveness, demonstrating that elicitor and light-mediated effects are achieved via the same DNA sequence element in the *CHS* promoter (Lozoya *et al.*, 1991). It is likely that the elicitor response pathway regulates the synthesis and/or the activation of relevant transcription factors that bind to the LRU. Support for this comes from the recent observation that fungal elicitors inhibit the light-induced accumulation of *CPRF1* transcripts in parsley cells (Hahlbrock *et al.*, 1995).

A similar type of differential regulation was observed in *Arabidopsis* cells with protein phosphatase inhibitors. That is, okadaic acid and cantharidin inhibit the UV/blue light induction of *CHS* (Fig. 4.7) whereas *PAL* is induced by phosphatase inhibitors, UV/blue light or both (Fig. 4.8 and 5.8). At equivalent concentrations,

okadaic acid and cantharidin also inhibited the UV/blue light-induced expression of an *Arabidopsis* LRU tetramer construct (Fig. 5.8) in transient expression assays with cell culture protoplasts (data not shown). Furthermore, studies with *Arabidopsis* cells reveal that *PAL* is induced by elicitor whereas *CHS* is not (Davis and Ausubel, 1989). It is therefore possible that protein phosphatase inhibitors activate the elicitor signalling pathway(s) inducing *PAL* and repressing *CHS* expression in the *Arabidopsis* cell culture. Consistent with this hypothesis, protein phosphatase inhibitors have been shown to mimic elicitor-mediated responses in a number of plant systems (Raz and Fluhr, 1993; Felix *et al.*, 1994; MacKintosh *et al.*, 1994; Mathieu *et al.*, 1996). For example, phosphatase inhibitors are reported to induce external alkalinisation and cytoplasmic acidification, an early event associated with elicitor recognition in plant cells (Felix *et al.*, 1994; MacKintosh *et al.*, 1994; Mathieu *et al.*, 1994; Mathieu *et al.*, 1996). The control of elicitor-induced extracellular and intracellular pH changes may concern proteins involved in the regulation of the cytosolic proton concentration, such as the plasma membrane proton-ATPase. Indeed, elicitor-induced changes of the phosphorylation state of the plasma-membrane proton-ATPase have been reported (Vera-Estrella *et al.*, 1994). In accordance with the above possibility, we have shown that cantharidin activates an early event in a, as yet unidentified, signalling pathway coupled to *PAL* gene expression in the *Arabidopsis* cell culture (Fig. 6.10). It is now important to establish whether fungal elicitors regulate *PAL* and *CHS* gene expression in the *Arabidopsis* cell culture. Pharmacological studies will then confirm whether cantharidin does in fact mediate its effects on flavonoid biosynthesis genes through an elicitor-mediated response pathway. It would also be of interest to determine whether there is an involement of jasmonic acid in such a pathway (Fig 3.8).

7.7 Conclusions

The work presented in this study enables several conclusions to be drawn about the UV/blue light signalling pathways regulating *CHS* gene expression in the *Arabidopsis*

cell culture system: (i) The UV/blue light induction of *CHS* involves at least two distinct UV-B and UV-A/blue phototransduction pathways. (ii) Both UV-B and UV-A/blue light signalling pathways require calcium, protein synthesis and protein phosphorylation. (iii) The UV-B and UV-A/blue pathways differ in the involvement of calmodulin. (iv) The same UV-B and UV-A/blue phototransduction pathways appear to regulate other flavonoid biosynthesis genes. (v) The UV-B and UV-A/blue light signal transduction pathways are different from the phytochrome signalling pathway regulating *CHS* in other species. (vi) UV-B and UV-A/blue light mediate their effects via transcription factors that bind to the LRU in the *CHS* promoter.

7.8 Future Work

Although this study has provided important information regarding the signalling processes involved in the UV-B and UV-A/blue light regulation of flavonoid biosynthesis gene expression, further experiments are required to examine other aspects of UV/blue light signal transduction. For example, since CRY1 contains a flavin chromophore (Ahmad and Cashmore, 1996), it is possible that redox reactions and electron transport are early steps in UV/blue light signal transduction. Recent studies have also shown that blue light activates a plasma membrane anion channel in *Arabidopsis* hypocotyl cells (Cho and Spalding, 1996). This anion channel activity is thought to be associated with the blue light inhibition of hypocotyl elongation, both of which are inhibited by the anion channel blocker, NPPB. However, similar concentrations of NPPB appeared to cause a general inhibition of gene expression in the *Arabidopsis* cell culture (see 4.10 and 4.11). Nevertheless, it is worth investigating the effects of other known anion channel blockers on the UV/blue light induction of *CHS* and *PAL* gene expression in the *Arabidopsis* cell culture. Examples include IAA-94 and niflumic acid, which have been shown to be effective anion channels blockers in *Vicia* guard cell protoplasts (Marten *et al.*, 1992). In addition, further experiments are required to establish whether H^+ and/or K^+ fluxes are involved in the UV/blue

phototransduction pathways, since the lack of effect of vanadate and tetraethylammonium on the UV-B and UV-A/blue light induction of *CHS* (Fig. 4.4A and B) may have been due to a lack of uptake.

While providing significant insights into the signalling processes concerned with the UV-B and UV-A/blue light induction of gene expression, *in vivo* experiments with pharmacological agents have their limitations. An important aspect of future research will be to employ cell physiological techniques to substantiate and extend the findings of this study. As mentioned already, transgenic *Arabidopsis* containing aequorin targeted to particular subcellular locations may prove useful in determining the nature of the calcium requirement for the UV-B and UV-A/blue phototransduction pathways. Furthermore, in relation to the ion channel inhibitor studies described above, electrophysiological studies with intact *Arabidopsis* plants or cells will establish whether UV/blue light can induce potential changes across the plasma membrane and which ion fluxes underlie such effects. Subsequent pharmacological experiments will determine whether such signalling events are associated with the induction of flavonoid biosynthesis gene expression.

The application of a genetic approach should lead to the isolation of mutants which are altered specifically in UV-B or UV-A/blue photoreception and signal transduction. The *Arabidopsis hy4* cell culture described here demonstrates the possibility of extending such an approach to the cellular level. It will be important to establish whether protoplasts isolated from this cell culture support high levels of transgene expression. This would provide a powerful system in which to investigate the structure-function relationship of the CRY1 protein and the primary signalling events associated with UV-A/blue light perception.

In a broader context, the cellular systems described here will also be useful for studying other signal-mediated responses, such as *CHS* induction by low temperature (Fig. 3.7). Moreover, the possible interaction of elicitor and UV/blue light-mediated responses in the *Arabidopsis* cell culture provides an excellent model for the study of 'cross-talk' between plant signal transduction pathways.

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