UV/blue light signal transduction regulating gene expression in *Phaseolus vulgaris* and *Arabidopsis thaliana*

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CONTENTS

Chapter 3: UV-A/blue light signal transduction regulating *rbcS*

Chapter 4: UV-A/blue and UV-B light signal transduction regulating *CHS* in *Arabidopsis*

Abbreviations

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

ATZ	3-amino-1,2,4-triazole
4CL	4-coumarate:CoA ligase
APX	ascorbate peroxidase
BSO	L-butathione-[S,R]-sulfoximine
$[Ca^{2+}]_{cyt}$	cytosolic calcium concentration
C4H	cinnamate 4-hydroxylase
CAB	LHCB1 chlorophyll-binding protein
CHI	chalcone isomerase
CHS	chalcone synthase
cop	constituitive photomorphogenic
cpm	counts per minute
CPRF	common plant regulatory factor
CRY	cryptochrome
DCPIP	dichlorophenol indophenol
DEPC	diethyl pyrocarbonate
det	de-etiolated
DFR	dihydroflavonol reductase
DMSO	dimethyl sulphoxide
DPI	diphenylene iodonium
DQ	duroquinone
EGTA	ethyleneglycol-bis-(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
FAD	flavin adenine dinucleotide
FDA	fluorescin diacetate
FeCN	potassium ferricyanide
fus	*fusca*
GSH	reduced glutathione
GST5	glutathione-S-transferase
GUS	β-glucuronidase
hy	long hypocotyl
H_2O_2	hydrogen peroxide

icx	increased chalcone synthase expression
MTHF	methenyltetrahydrofolate
NAC	N-acetyl-L-cysteine
nph	non-phototropic hypocotyl
Oac	acetate
OD	optical density
O_2^-	superoxide
PAL	phenylalanine ammonia-lyase
PDTC	pyrrolidinedithiocarbamate
3-PG	3-phosphoglycerate
pH	hydrogen ion concentration ($-\log_{10}$)
PMS	phenazine methosulfate
PR	pathogenesis related
psi	pounds per square inch
rbcS	ribulose-1,5-bisphosphate carboxylase small subunit
ROS	reactive oxygen species
SA	salicylic acid
35S	cauliflower mosaic virus 35S promoter
TCH	touch
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
UV-A	320-390 nm
UV-B	280-320 nm
UV-C	wavelengths below 280 nm
v/v	volume/volume
W-7	N-(6-aminohexyl)5-chloro-1-napthalenesulphonamide
w/v	weight/volume

SUMMARY

Plants regulate the expression of some genes in response to ultraviolet (UV) and blue light. To investigate the signal transduction pathways regulating UV/blue light regulated gene expression, the effects of specific inhibitors were examined in protoplasts isolated from dark-adapted *P. vulgaris* leaves and a photomixotrophic *Arabidopsis* cell culture.

Pharmacological studies with *P. vulgaris* protoplasts indicate that calcium, protein phosphorylation and dephosphorylation and protein synthesis are required for UV-A/blue light regulation of genes encoding the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (rbcS), which is similar to the UV-A/blue and UV-B light signalling components regulating chalcone synthase (*CHS*) expression in *Arabidopsis* cells. Further studies were carried out in *Arabidopsis* cells to characterise the UV-A/blue and UV-B regulation of *CHS*. A 5 min UV-B illumination, followed by transfer to non-inductive, low fluence rate white light for 6 hours, was sufficient to induce *CHS* transcripts. In contrast, a one hour UV-A/blue illumination, followed by transfer to non-inductive light for 5 hours was required before any *CHS* transcript accumulation was detected. This indicates that the two pathways are distinct. However, both the UV-A/blue and UV-B light regulation of gene expression appears to involve plasma membrane redox activity, because the impermeable electron acceptor ferricyanide (FeCN), strongly inhibited UV-A/blue and UV-B phototransduction. Additionally, the flavoprotein inhibitor diphenylene iodonium (DPI), strongly inhibited UV-A/blue and UV-B induced *CHS* and *PAL* expression. These results suggest that the phototransduction pathways require at least one flavoprotein-mediated electron transfer step as a signalling component.

Expression of the *Arabidopsis* gene encoding the calmodulin-like protein TOUCH3 (TCH3) is induced by FeCN and DPI. Cells treated with a calcium ionophore were not altered in UV/blue light regulated *CHS* expression. This

indicated that cytosolic calcium increases induced by FeCN and DPI are not inhibiting UV/blue phototransduction. However, UV-A/blue and UV-B light inhibited the ionophore-induced expression of *TCH3* in *Arabidopsis* cells. This implied that UV-A/blue and UV-B light were activating a calcium efflux mechanism, lowering cytosolic calcium concentrations. Therefore, the effect of an inhibitor of calcium-ATPases, erythrosin B (EB), was examined. EB prevented the UV-A/blue light induction of *CHS*. However, EB had no effect on the UV-B induction of *CHS*. Further pharmacological studies were carried out to characterise the UV/blue inhibition of *TCH3* expression in the presence of ionophore.

Transgenic *Arabidopsis* wild-type and *hy4* mutant plants expressing the cytosolic calcium reporter protein, aequorin, were generated. UV-A/blue light induced an increase in aequorin luminescence in both transgenic lines, indicating that UV-A/blue light induces an increase in cytosolic calcium concentration, but this response is not mediated by CRY1.

No effect on the regulation of *CHS* expression by reactive oxygen species (ROS) and scavengers of ROS was observed, suggesting that the production of ROS by plasma membrane redox processes is not a component of the signalling pathways. Interestingly, UV-A/blue and UV-B light strongly induced *GST5* transcripts in the cell culture and plants. The experiments described in this thesis are discussed and a hypothesis for the signal transduction processes involved in UV/blue regulated gene expression is presented.

CHAPTER 1: UV/BLUE LIGHT PHOTOPERCEPTION AND SIGNAL TRANSDUCTION IN PLANTS

1.1 Introduction

Light provides essential signals which higher plants use to control their behaviour, metabolism and development. Regulation of plant responses to light is mediated by several different types of photoreceptors. Light signals perceived by these photoreceptors are transduced via signalling components to bring about diverse downstream physiological responses. In many cases responses have been shown to involve the transcriptional regulation of specific genes.

Responses to red and far-red light are mediated by the photoreceptor phytochrome (Furuya, 1993). Phytochrome is a well-characterised cytosolic protein dimer consisting of two approximately 120 kDa polypeptides, each carrying a covalently bound linear tetrapyrrole chromophore. Phytochrome exists in two photointerconvertible forms, one (Pr) absorbs red light, and upon absorption of red light converts to Pfr, which is generally considered to be the biologically active form of phytochrome. Pfr absorbs far-red light, and upon illumination with far-red light is converted to Pr. In most higher plants phytochrome is encoded by small gene families, in *Arabidopsis* five phytochrome genes have been identified (Sharrock and Quail, 1989). Considerable attention has been paid to the multitude of responses regulated by phytochrome. However, light in the near UV (UV-A, 320-390 nm) and blue (390-500 nm) regions of the spectrum also controls plant developmental responses, including stem extension, leaf development, phototropism and transcription of various genes. UV-B radiation (280-320 nm)

induces several responses, notably the production of UV-absorbing protective pigments.

In comparison to phytochrome signal perception and transduction, there is less information on UV/blue light photoperception and signal transduction processes. UV/blue photoreceptors have not been fully characterised and our knowledge of UV/blue phototransduction mediating the responses is fragmentary, particularly regarding the UV/blue light regulation of gene expression. This chapter reviews recent advances in UV/blue photoperception and signal transduction pathways in higher plants

1.2 UV/blue photoperception

Numerous responses are regulated by the near UV-A and blue regions of the spectrum, including gene expression (Kaufman, 1993; Short and Briggs, 1994; Jenkins *et al.*, 1995). For example, UV-A/blue light regulates transcription of genes encoding the small subunit of the photosynthetic enzyme, ribulose 1,5-bisphosphate carboxylase oxygenase (rbcS) (Sawbridge *et al.*, 1994; Conley and Shih, 1995). The reactions catalyzed by rubisco are shown in Figure 1.1. Additionally, UV-A/blue light regulates expression of the genes encoding enzymes of the phenylpropanoid pathway, including chalcone synthase (CHS) and phenylalanine ammonia-lyase (PAL) (Kubasek *et al.*, 1992; Mol *et al.*, 1996). The reactions catalyzed by CHS and PAL are shown in Figure 1.2.

Action spectra for a wide range of different responses to UV and blue light have been obtained. Studies of action spectra and experiments with flavin inhibitors indicate that the most likely candidate for the chromophore of a blue light photoreceptor is a flavin (Briggs and Iino, 1983; Galland and Senger, 1988a). However, there is also evidence that pterins can function in UV/blue photoreception in particular systems (Galland and Senger, 1988b). Only in recent

Figure 1.1 Reactions catalyzed by Rubisco

The reactions catalyzed by Rubisco are shown in the following Figure. Rubisco catayzes the addition of CO_2 to ribulose 1,5 bisphosphate to form 3-phosphoglycerate (3-PG) . Rubisco is also an oxygenase, catalyzing the addition of O_2 to ribulose 1,5-bisphosphate to form phosphoglycolate and 3-PG. Phoshphoglycolate is salvaged into glycolate which enters peroxisomes and is subsequently oxidized to glycolate. Transamination of glyoxalate yields glycine. 3-PG enters the Calvin cycle where it is converted into fructose 6-phosphate and other hexose sugars, and the Rubisco substrate, ribulose 1,5-bisphosphate, is regenerated.

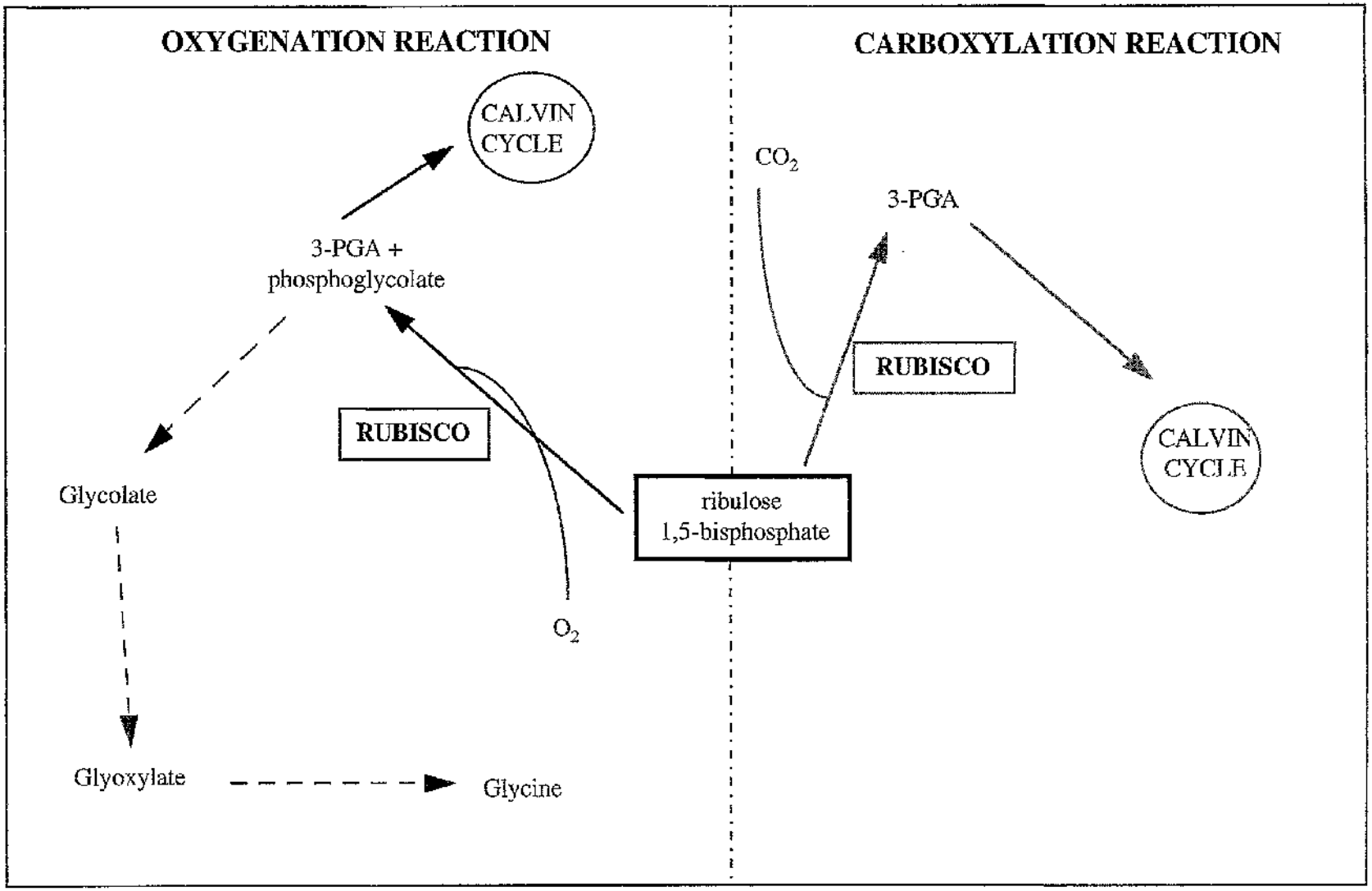
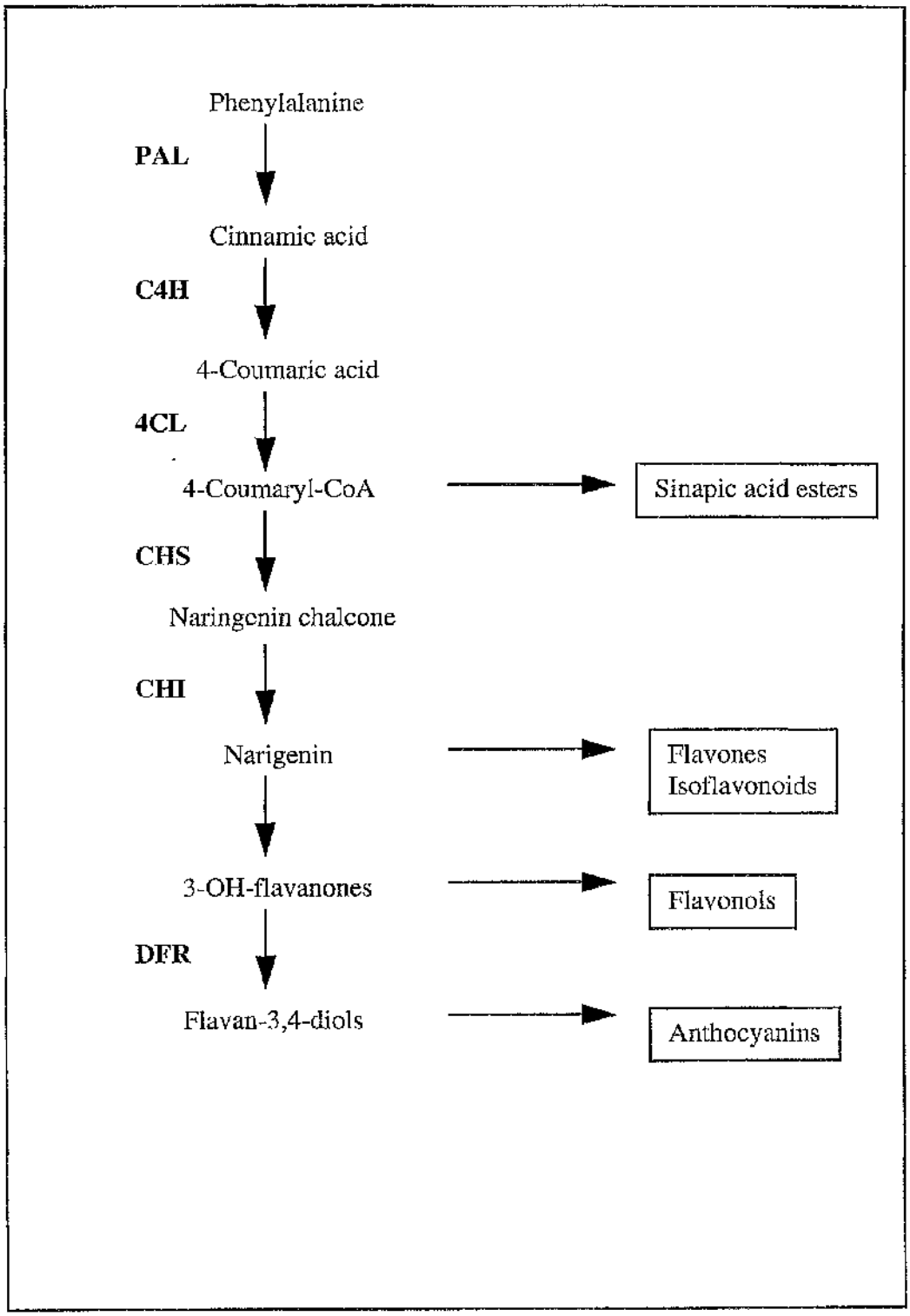

OXYGENATION REACTION
CARBOXYLATION REACTION
CALVIN CYCLE
3-PGA + phosphoglycolate
RUBISCO
Glycolate
Glyoxylate
Glycine
O_2
ribulose 1,5-bisphosphate
CO_2
3-PGA
RUBISCO
CALVIN CYCLE

Figure 1.2 Reactions catalyzed by enzymes of the phenylpropanoid and flavonoid biosynthesis pathway

The reactions catalyzed by phenylalanine ammonia-lyase (*PAL*), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI) and dihydroflavonol reductase (DFR) are shown in the following figure. PAL catalyzes the initial step of these core reactions, CHS catalyzes the first committed enzyme of flavonoid 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

years has any real progress been made in identifying UV/blue photoreceptors. These are discussed below.

1.2.1 Cryptochrome photoreceptor

1.2.1.1 The *Arabidopsis* CRY1 photoreceptor

The *Arabidopsis hy4* mutant was isolated by Koornneef *et al.* (1980) in a white light screen, due to its extended hypocotyl compared to wild-type. Unlike the other *hy* mutants, hypocotyl growth suppression in *hy4* was not impaired in red or far-red light, but in blue and UV-A light (Koornneef *et al.*, 1980; Ahmad and Cashmore, 1993), indicating that *hy4* was specifically altered in UV-A/blue light perception. Further characterisation revealed that the *hy4* mutant has a longer hypocotyl phenotype in UV-A/blue and green light (Jenkins *et al.*, 1995)

The *HY4* gene was cloned by gene tagging from T-DNA lines (Ahmad and Cashmore, 1993). The N-terminal region of the derived amino acid sequence was found to have striking homology to microbial DNA photolyases (Ahmad and Cashmore, 1993). Cashmore and co-workers subsequently renamed the HY4 protein CRY1, after cryptochrome, the term used previously to describe plant UV-A/blue photoreceptors (Ahmad and Cashmore, 1996). Interestingly, CRY1 showed significant homology in the C-terminus to rat smooth muscle tropomyosin A. The significance of this is not known, although the CRY1 sequence is not conserved in regions of α-helices predicted from the Chou-Fasman parameters for tropomyosin (Ahmad and Cashmore, 1996). Therefore, this domain of CRY 1 could be involved in protein-protein interactions.

DNA photolyases are a unique class of flavoproteins that function in response to light to repair damaged DNA by donating electrons to pyrimidine dimers in the DNA (Sancar, 1994). Photolyases bind two chromophores. Flavin

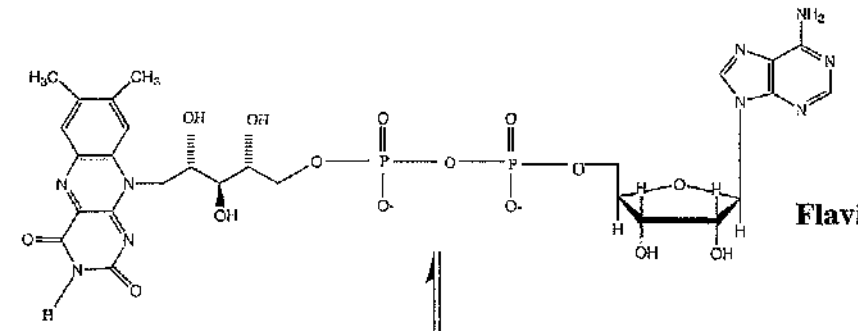
adenine dinucleotide (FAD) is attached at the C-terminus and donates electrons to the pyrimidine dimers. The structure of FAD is shown in Figure 3.1. The second N-terminal chromophore acts in light harvesting and transfer of electrons to the FAD chromophore. Three classes of photolyases have been characterised, distinguished by absorption properties and the light-harvesting chromophore attached at the N-terminal domain. Long-wave photolyases with maximum absorption in the blue (around 450 nm) bind 5-deazaflavin as the light-harvesting chromophore, short and medium wavelength photolyases with maximum absorption in the UV-A region (350-370 and 410 nm respectively) typically bind a pterin (Sancar, 1994). The light-harvesting chromophore absorbs UV/blue light and transfers an electron to the flavin chromophore which donates the electron to the pyrimidine dimer, repairing the damaged DNA.

The photoreceptor protein, CRY1, was characterised at the protein level (Lin *et al.*, 1995a). CRY1 purified from expression in baculovirus-infected cells was observed to bind flavin adenine dinucleotide (FAD) non-covalently. Interestingly, CRY1 is capable of mediating green light responses (Lin *et al.* 1995a), a property not shared by *E.coli* photolyase. This is most likely to be a direct result of the redox properties of the flavin which binds to CRY1. As well as forming the two different states, FAD and $FADH_2$, the flavin is stable in the neutral radical flavosemiquinone form, $FADH^{\cdot}$ (Lin *et al.*, 1995a). This semiquinone form absorbs green light.

Malhotra *et al.* (1995) further identified the two chromophores of CRY1. A fusion protein between the 'photolyase' domain of CRY1 and maltose-binding protein was expressed in *E.coli* and purified. The absorption spectrum of CRY1 peaked at 410 nm. Fluoresence emission spectra detected two peaks, one at 460 nm, which is consistent with the pterin, methenyltetrahydrofolate (MTHF). The structure of the E.coli photolyase cofactor, 5,10- MTHF is shown in Figure 1.3. The second peak at 520 nm, is consistent with FAD.

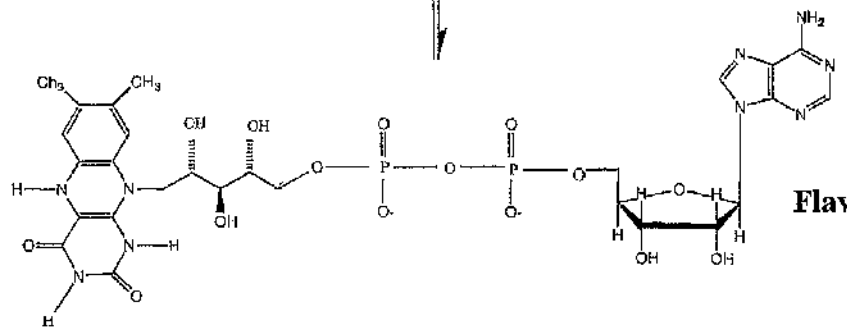
Figure 1.3 Structure of flavin and MTHF

The structure of two photolyase chromophores FAD and 5,10-MTHF are shown opposite.

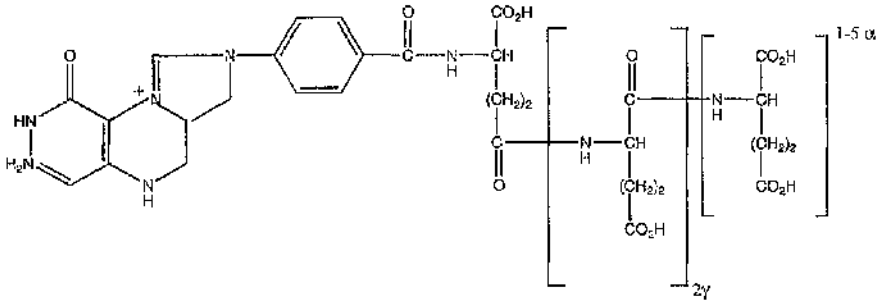


Flavin adenine dinucleotide
Oxidised form
(FAD)

$-H_2$ $+H_2$



Flavin adenine dinucleotide
Reduced form
($FADH_2$)



5,10- MTHF

Although MTHF is usually associated with short-wavelength photolyases absorbing in the near UV region, recently a medium-wavelength photolyase was characterised that binds pterins and has an absorption maximum shifted to blue peaking at 410 nm, the same as CRY1 (Ahmad and Cashmore, 1996). However, the precise nature of the CRY1 second chromophore in *Arabidopsis* remains to be determined.

CRY1 lacks any detectable photolyase activity , and also lacks a tryptophan residue found in photolyases believed to be important for binding to pyrimidine dimers (Ahmad and Cashmore, 1993; Lin *et al.*, 1995a). CRY 1 was predicted to be a soluble protein from its sequence which contained no membrane spanning domains. Fractionation experiments revealed that the majority of CRY 1 was associated with the soluble fractions, though a small proportion was also membrane associated (Ahmad and Cashmore, 1996). CRY 1 transcript and protein levels do not appear to be regulated by light (Ahmad and Cashmore, 1993; 1996).

1.2.1.2 CRY1-mediated responses

The long hypocotyl phenotype of *hy4* demonstrates that CRY1 is involved in controlling hypocotyl extension. In addition, CRY1 controls the UV-A/blue light induction of anthocyanin formation by regulating the level of transcription of *CHS* and genes encoding other flavonoid biosynthetic enzymes (Chory, 1992; Ahmad *et al.*, 1995; Jackson and Jenkins, 1995). Furthermore, CRY1 is required for full expression of certain nuclear-encoded chloroplast protein genes, including *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. Furthermore, CRY1 appears to be required for apical hook opening (Liscum and Hangarter, 1993).

Overexpression studies can be useful in determining the function of a protein. Overexpression of the *HY4* gene in transgenic tobacco or *Arabidopsis* resulted in plants hypersensitive to UV-A, blue and green light; the plants showed an enhanced hypocotyl suppression response and increased production of anthocyanin (Lin *et al.*, 1995b). These enhanced responses were not observed under red or far-red light. 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.

1.2.1.3 Mechanism of CRY1 action

CRY1 protein does not show photolyase activity, although the protein is very similar to microbial DNA photolyases. The most likely mode of action of CRY1 is, like photolyase, mediating a UV-A/blue and green light dependent electron transfer, presumably not to DNA but to a downstream partner. The downstream partner would then be activated by this reduction. A possible function of the the C-terminus would be to bind this downstream partner.

1.2.1.4 Additional cryptochrome photoreceptors

One additional CRY1-related sequence has been identified in the *Arabidopsis* genome. 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 (Lin *et al.*, 1996). Overexpression

of CRY2 in transgenic plants suggests that CRY2 possesses properties related to, but distinct from, CRY1. Transgenic plants displayed a small enhancement of the hypocotyl suppression response, an increased sensitivity of cotyledon expansion to blue light but little effect on anthocyanin production (Cashmore, 1997). Therefore, CRY2 functions as a photoreceptor possessing related but distinct properties to CRY1.

A putative blue light photoreceptor gene, *SA-phr1*, was isolated from *Sinapis alba*. Using synthetic oligonucleotides with sequence corresponding to a highly conserved region in the carboxy-terminus of microbial photolyases, Batschauer and co-workers screened a cDNA library and isolated the *SA-phr1* clone (Batschauer, 1993). It was found that the gene encoding the putative blue light photoreceptor has very high homology to microbial DNA photolyases. Although at first the *SA-phr1* gene was believed to encode DNA photolyase, (Batschauer, 1993), more detailed studies were able to show that, like CRY1, SA-phr1 has no photolyase activity, and is most likely to function as a blue light photoreceptor in *S.alba* (Malhotra *et al.*, 1995). In contrast to CRY1, the *SA-phr1* transcript is light-regulated (Batschauer, 1993). However, Cashmore (1997) noted that SA-phr1 lacks a C-terminal domain characteristic of CRY1 and CRY2, and no sequence equivalent exists in *Arabidopsis*. Thus the photolyase/cryptochrome in *S. alba* is distinct from those in *Arabidopsis*.

Other cryptochrome-like sequences have been identified in *Chlamydomonas* and fern, although the role of these putative photoreceptors is not known (Cashmore, 1997).

1.2.2 The photoreceptor for phototropism

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).

1.2.2.1 Genetic analysis of phototropism

Mutants of *Arabidopsis* specifically altered in phototropism (JK218, JK224 and JK229) were described by Khurana and Poff (1989). JK224 is of particular interest. JK224 exhibited a 20-30 fold shift in the fluence threshold, but no change in magnitude, for blue-light-dependent first-positive curvature and was unaltered in second-positive curvature. Khurana and Poff (1989) proposed that the mutation was likely to be at the level of the photoreceptor. Meanwhile, 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. These *n*on-*p*hototropic *h*ypocotyl (*nph*) mutants were found to represent four genetic loci, *nph1* to *nph4*. 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 lacked both first and second-positive phototropism in response to blue and green light.

1.2.2.2 A blue-light induced membrane phosphoprotein regulating phototropism

Short and Briggs (1990) observed that illumination of isolated pea membranes caused an increase in phosphorylation of a 120 kDa protein. Short *et al*. (1992) proposed that blue light exposes sites in the 120 kDa protein which are subsequently phosphorylated. Similar blue light induced phosphorylation effects

have been observed in maize (Hager *et al.*, 1993; Palmer *et al.*, 1993) and *Arabidopsis* (Reymond *et al.*, 1992a). Blue light dependent phosphorylation of a maize plasma membrane protein was demonstrated to be redox regulated, and Hager *et al.* (1993) proposed that blue light dependent reduction of the protein may expose phosphorylation sites on the protein which can either be autophosphorylated, or phosphorylated by a separate kinase activity.

Blue light regulated phosphorylation of the 120 kDa plasma membrane protein has been proposed to mediate phototropism in pea (Short and Briggs, 1990; 1994). The fluence, time dependence and tissue location of the blue light induced phosphorylation response is compatible with first-positive phototropism. A similar correlation has been made in maize (Palmer *et al.*, 1993). Therefore, Short and Briggs (1994) proposed that the phosphorylation event was likely to be an early step in the phototropism signal transduction pathway.

Genetic analysis further linked the phosphoprotein to phototropism. The phototropic mutant JK224 (*nph1-2*) was found to be defective in the blue-light phosphorylation reaction as well as blue-light inducible first-positive curvature (Reymond *et al.*, 1992b). Liscum and Briggs (1995) found that all *nph1* mutants completely lacked blue light-dependent protein phosphorylation. Therefore, the 124 kDa plasma membrane associated phosphoprotein appears to be NPH1 and probably functions as a photoreceptor for phototropism. Although *nph2, 3* and *4* are impaired in phototropism, normal amounts of the phosphoprotein were present, and light-inducible phosphorylation was similar to wild-type (Briggs and Liscum, 1997). Therefore, NPH2, 3 and 4 probably function downstream of NPH1. Additionally, *nph4* is gravitropically impaired, and NPH4 probably functions in a shared gravitropism and phototropism signalling pathway.

As NPH1 is most likely the photoreceptor regulating phototropism, there has been particular interest in the *nph1* mutant. Particularly interesting is the identification of the chromophore regulating NPH1 phosphorylation. The mutant JK224 (*nph1-2*), was generated by EMS mutagenesis, resulting in a point

mutation. JK224 is only altered in first-positive curvature, unlike the NPH1 null mutant which is deficient in all photropic responses (Briggs and Liscum, 1997). It has been proposed that the point mutation alters binding of one chromophore in JK224, but the activity of the protein is unaltered in other aspects of phototropism. Therefore Briggs and Liscum (1997) proposed that the chromophore regulating NPH1 phosporylation may be a dual chromophore photoreceptor, analogous to CRY1.

Genetic analysis has demonstrated that CRY1 is distinct biochemically and functionally from NPH1. *hy4* has normal phototropism (Liscum and Briggs, 1995) and light-inducible phosphorylation (Briggs and Liscum, 1997), whereas *nph1* has normal suppression of hypocotyl elongation. Additionally, NPH1 appears to be a plasma membrane protein, whereas CRY1 is a soluble protein. A double mutant displays neither phototropism or blue-light suppression of hypocotyl elongation (Briggs and Liscum, 1997). Cloning and sequencing of the gene for NPH1 will reveal the nature of the protein, whether it is likely to bind two chromophores and whether it has a kinase activity.

1.2.3 UV-B perception

In plants, UV-B irradiation has a number of damaging effects (Tevini and Teramura, 1989; Stapleton, 1992). 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). UV-B induces the expression of genes encoding enzymes concerned with flavonoid biosynthesis and hence increases the levels of UV-absorbing pigments. Furthermore, the induction of *CHS* gene expression by UV-B light is distinct to induction by UV-A/blue light. Fuglevand *et al.* (1996) showed that CRY1 does

not mediate the UV-B induction of *CHS* in *Arabidopsis*, wheras it does mediate UV-A/blue light induction. It is therefore likely that the UV-B induction of gene expression is mediated by photoreceptors absorbing specifically UV-B and that photoreception is coupled to transcription through a cellular signalling pathway.

There is little known about the nature of the UV-B photoreceptor(s) (Jenkins *et al.*, 1997). Flavins have been proposed as chromophore pigments (Galland and Senger, 1988a, 1988b). Support for this 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 Schafer, 1992). Pterins are also strong candidates for the chromophore of a UV-B photoreceptor (Galland and Senger, 1988b). One approach to identify UV-B photoreceptors is a genetic analysis.

1.3 UV-A/blue and UV-B signalling components

The analysis of components of plant phototransduction pathways has been an area of increasing interest. A biochemical approach, primarily taking advantage of knowledge about mammalian cell signalling pathways, has revealed information on the phytochrome signalling pathways regulating gene expression (Quail, 1994). In comparison, there is not as much information on components of UV/blue phototransduction pathways regulating gene expression (Jenkins *et al.*, 1995; Short and Briggs, 1994; Kaufman, 1993). This section reviews information on UV/blue light signalling components identified by biochemical analysis, some of which have been demonstrated to regulate gene expression.

1.3.1 Plasma membrane potential and ion fluxes

Plasma membranes of plant cells contain ion channels and pumps which respond to environmental stimuli, including ABA and elicitors, resulting in fluxes of the important ions, Ca^{2+}, K^+, Cl^- and H^+ across the plasma membrane, altering membrane potentials and voltage gated ion channels (Ward *et al.*, 1995; Maathuis and Sanders, 1992). Several rapid UV/blue light induced effects on plant plasma membranes have been identified using electrophysiological techniques.

Perhaps the best-studied example of blue light activated ion fluxes and electrical changes is the rapid response of etiolated hypocotyls. Spalding and Cosgrove (1988) described a large, rapid depolarization of the plasma membrane of etiolated cucumber (*Cucumis sativus* L.) hypocotyls on exposure to blue light. The depolarization, 100mV, precedes growth inhibition. Spalding and Cosgrove (1992) proposed that the depolarization was a result of inhibition of the plasma membrane H^+-ATPase. Repolarization was proposed to result from the inward movement of Ca^{2+}, raising intracellular calcium levels which inactivate K^+ channels and resume H^+ pump activity. However, inhibition of the H^+-ATPase was proposed to be insufficient alone to account for the membrane depolarization, and it was proposed that anion channel activity is involved. Subsequent studies with *Arabidopsis* seedlings demonstrated blue light activation of an anion channel (Cho and Spalding, 1996). This activity was inhibited by the anion channel inhibitor, 5-nitro-2,3-phenylpropylaminobenzoic acid (NPPB). NPPB also inhibited the blue light induced membrane depolarization and reduced blue light regulated hypocotyl growth suppression. The authors proposed that anion channel activity plays a role in transducing blue light to growth inhibition.

Nishizaki (1988) demonstrated that a blue light induced depolarization of the pulvinar motor cell plasma membrane potential preceeded movement of primary

leaves of *Phaseolus vulgaris*. Nishizaki (1994) reported that the blue light induced plasma membrane depolarization in *Phaseolus vulgaris* may involve an inactivation of the plasma membrane H^+-ATPase because depolarization was reduced by inhibitors of H^+-ATPase activity.

Blue light has been shown to induce H^+ pumping in guard cells during stomatal opening (Assmann *et al.*, 1985). This H^+ pumping results in hyperpolarization of the plasma membrane, opening K^+-selective channels allowing passive K^+ uptake. There is some argument over whether the activation of H^+ pumping is due to a direct effect on the H^+ATPase or due to plasma membrane redox activity, as discussed below.

1.3.2 Plasma membrane electron transport

Electron transport not only occurs in the mitochondria, chloroplasts and endoplasmic reticulum, but also at the plasma membrane of plants cells (Rubinstein and Luster, 1993). There is much evidence now that light-responsive redox components are present in plant plasma membranes and blue light regulated plasma membrane redox processes have been demonstrated in a number of different plant species.

A well characterised response is the blue light regulated reduction of a b-type cytochrome. b-type cytochromes have been identified in plasma membranes of both green and etiolated tissue, in many different organs of different plant species (Asard *et al.*, 1994) The b-type cytochrome of the plasma membrane is a high-redox (generally between +100 and +165 mV) potential protein, which is rapidly reducible by ascorbate (+80 mV) and readily oxidized by artificial electron acceptors, ferricyanide (+360 mV) and dichlorophenol indophenol (DCPIP) (+210 mV). Blue light illumination of plasma membrane fractions results in a transient reduction of the b-type cytochrome. The action spectrum and inhibitor studies

point to the action of a flavin-like chromophore in blue light mediated b-type cytochrome reduction (Asard *et al.*, 1994; 1995). However, there is presently no evidence that the b-type cytochrome has an *in vivo* physiological function. Purification of the protein may lead to the identification of a role for the cytochrome in plant responses.

Blue light induces H^+ extrusion from guard cells leading to acidification of the external medium. Gautier *et al.* (1992) demonstrated that ferricyanide, an external electron acceptor, reduced H^+ pumping in *Commelina communis L.* guard cell protoplasts. They proposed that ferricyanide was competing with blue light regulated plasma membrane redox activity. Therefore, it appears that H^+ pumping may be regulated by blue light induced plasma membrane electron transport. Further research is needed to establish the role of the redox system and the H^+ - ATPase in H^+ efflux.

Dharmawardhane *et al.* (1989) demonstrated blue light stimulated plasma membrane electron transport in *Avena* leaf mesophyll cells, by measuring ferricyanide reduction in the bathing solution. They found that sphingoid bases (sphingosine derivatives), which are potent inhibitors of protein kinase C in animal cells, stimulated redox activity in blue light, but inhibited the effect in darkness. They proposed a model incorporating two redox systems, one stimulated by blue light and inactivated by a sphingoid base-sensitive phosphorylation.

More recently, Berger and Brownlee (1994) proposed that blue light regulation of the direction of rhizoid outgrowth in germinating *Fucus* zygotes is mediated by a blue light stimulated plasma membrane redox chain. Ferricyanide inhibits the directional component of this response, but not germination *per se*.

1.3.3 Reactive oxygen species

Although reactive oxygen species (ROS) are principally associated with pathogen signalling pathways in plants (Lamb and Dixon, 1997), recently there has been interest in the role of light, particularly UV-B light, in generation of ROS as a component of signalling pathways.

Recently Rao *et al.* (1996) proposed that UV-B light induced ROS. They found that there was a significant increase in NADPH-oxidase activity after one day of UV-B light treatment in *Arabidopsis* plants. Green and Fluhr (1995) reported that UV-B induces transcript and protein accumulation of the pathogenesis associated protein, PR-1, in tobacco leaves. The antioxidants N-acetyl-L-cysteine and pyrrolidinedithiocarbamate inhibited UV-B induced *PR-1* accumulation, whereas rose bengal, a generator of ROS, induced an accumulation of PR-1 in the absence of UV-B light. The authors therefore proposed that the signalling pathway coupling UV-B perception to PR-1 accumulation is mediated by ROS.

Shinkle and Jones (1988) reported that blue light regulated hypocotyl growth suppression is regulated by a cell wall peroxidase. They found that ascorbate prevented the blue light induced inhibition of elongation. Additionally, peroxidase activity isolated from the cell wall was inhibited by ascorbate. They proposed that a cell wall localized peroxidase was mediating a decrease in wall extensibility. Shinkle and Jones (1988) proposed that the plasma membrane electron transport chain could supply reductant for enzymes in the cell wall.

1.3.4 G-proteins

Warpeha *et al.* (1991) reported that a brief illumination of low fluence blue light stimulates a transient increase in GTPase activity in plasma membrane enriched fractions from etiolated pea apical buds. Inhibitors of G-proteins completely

abolished the blue light mediated GTPase activity. Blue light irradiation stimulated binding of the radiolabelled GTP analogue, GTP[γ^{35}S] to the pea plasma membrane fraction. Using antibodies raised against mammalian G-protein α-subunits, a 40 kDa polypeptide was identified. This size of polypeptide is consistent with that of α-subunits in animal cells. Thus, the 40 kDa protein could be a α-subunit of a G-protein involved in a blue light signalling pathway. Warpeha *et al.* (1992) went on to demonstrate that flavin antagonists, PAA and KI, inhibited the blue light stimulated GTPγS binding to the pea membrane protein. This indicates that the photoreceptor involved in the light-induced binding of GTP may contain a flavoprotein. However, a physiological role for the blue light activated G-protein activity is not currently known.

1.3.5 Calcium and calmodulin

Calcium is known to be an important second messenger in plant cells (Bush, 1995). For calcium to act as a second messenger in plant signal transduction pathways, the plant cell must fulfil the following criteria: (i) a tightly regulated concentration of cytosolic calcium; (ii) calcium influx channels and efflux transporters in plasma membrane and internal membranes; and (iii) calcium regulated proteins, such as calmodulin (CaM) and Ca^{2+}/CaM dependent kinases. Plant cells have a resting cytosolic calcium concentration of approximately 100 nM, contain calcium channels and pumps at both the plasma membrane and internal membranes and several calcium binding proteins have been identified (Bush, 1993, 1995; Roberts and Harmon, 1992; Poovaiah and Reddy 1993; Johannes *et al.* 1991).

Changes in cytosolic calcium occur in plant cells in response to a variety of stimuli including cold shock, touch, oxidative stress and fungal elicitors (Bush, 1995). In addition, several studies have indicated a role for calcium and calcium regulated proteins in UV/blue signalling pathways.

UV-A and blue light induced chloroplast movement in the green alga *Mougeotia scalaris* is regulated by calcium (Russ *et al.*, 1991). UV-A and blue light increase intracellular calcium, as measured by the fluorescent calcium dye, Indo-1. Additionally, the calcium agonist Bay-K8644 and the ionophore A23187 increased chloroplast movement. Russ *et al.* (1991) proposed that an increase in cytoplasmic calcium, mediated by UV-A and blue light, results in a decrease of cytoplasmic viscosity, possibly by microtubule depolymerization, resulting in increased chloroplast movement.

Low fluence UV radiation results in loss of K^+ from cultured rose cells (Murphy and Wilson, 1982). The UV stimulated response was inhibited by the calcium chelator, EGTA, and by the calcium channel blocker, lanthanum (Murphy, 1988). Shinkle and Jones (1988) also used EGTA to implicate calcium in blue light inhibition of hypocotyl elongation in cucumber seedlings. EGTA suppressed the blue light response, and this could be recovered by addition of calcium. Other divalent cations did not restore the blue light-induced inhibition of elongation.

The first evidence for an involvement of calcium in UV/blue regulated gene expression was recently described (Christie and Jenkins, 1996). They demonstrated that UV-B and UV-A/blue light signal transduction pathways regulating *CHS* expression in *Arabidopsis* cells were inhibited by the calcium channel blockers, nifedipine and ruthenium red. Artificially increasing cytosolic calcium, however, did not stimulate *CHS* expression. Christie and Jenkins (1996) proposed that although calcium is required for UV-A/blue and UV-B regulated *CHS* expression, a calcium increase is not sufficient to stimulate *CHS* expression in *Arabidopsis* cells. Recently, calcium channel blockers have been used to

implicate calcium in the UV-B light induction of *CHS* and *PAL* expression in parsley protoplasts (Frohnmeyer *et al.*, 1997).

Calmodulin, a calcium-regulated protein, has been impicated in several UV/blue responses. Blue light induced H^+ pumping, regulating stomatal closure in guard cells of *Vicia faba* L., was inhibited by the calmodulin inhibitors, W-7 and trifluoperazine (Shimazaki *et al.*, 1992).

UV-B regulated *CHS* expression in *Arabidopsis* cells appears to require calmodulin, as *CHS* transcript accumulation in UV-B light was inhibited by W-7. In contrast, UV-A/blue light regulated *CHS* expression was not inhibited by W-7. Therefore, calmodulin was proposed to mediate UV-B but not UV-A/blue light regulated *CHS* expression in *Arabidopsis*. This demonstrates that UV-A/blue and UV-B signalling pathways regulating *CHS* are distinct in *Arabidopsis* cells. Additionally, UV-B light regulated *CHS* and *PAL* expression in parsley protoplasts is inhibited by W-7 (Frohnmeyer *et al.*, 1997).

1.3.6 Protein kinases and phosphatases

A number of plant protein kinases have been identified and characterised (Roberts and Harmon, 1995). The best studied example of UV/blue light regulated protein phosphorylation is of the plasma membrane protein mediating phototropism (Briggs and Liscum, 1997). This is discussed in Section 1.2.5. Additionally, blue light mediated protein phosphorylation has been shown to be regulate H^+ pumping in stomatal guard cells (Shimazaki *et al.*, 1992). H^+ pumping was prevented by inhibitors of the Ca^{2+}/CaM-dependent myosin light chain kinase (MLCK). Therefore, a MLCK -like protein appears to be involved in the signal transduction pathway coupling blue light photoreception to H^+ efflux in stomatal guard cells.

Light induced expression of *rbcS* and *C4 pyruvate orthophosphate dikinase* (*C4ppdkZm1*) genes in a maize protoplast transient assay system was inhibited by

the protein phosphatase inhibitors, okadaic acid and calyculin A (Sheen, 1993). Therefore, protein dephosphorylation regulated by light is coupled with gene expression in maize protoplasts.

Protein phosphorylation/dephosphorylation has also been implicated in UV/blue light regulated gene expression. Christie and Jenkins (1996) demonstrated that UV-A/blue and UV-B light regulated *CHS* transcript accumulation in *Arabidopsis* cells was inhibited by the serine/threonine protein kinase inhibitors staurosporine and K252a, whereas the tyrosine protein kinase inhibitor, genistein had no effect. The protein phosphatase inhibitors cantharidin and okadaic acid also inhibited UV/blue light regulated *CHS* expression. Frohnmeyer *et al.* (1997) recently reported the inhibition of UV-B regulated *CHS* and *PAL* transcripts in parsley protoplasts by serine/threonine, but not tyrosine, kinase inhibitors.

1.4 UV/blue photoregulatory mutants

Recently, downstream components of phytochrome signalling pathways have been identified using a genetic approach (Whitelam *et al.*, 1993; Barnes *et al.*, 1996). This section reviews recently identified mutants which are altered in components involved in UV/blue light regulated gene expression.

1.4.1 *Arabidopsis ICX* mutants

UV/blue light regulates genes of flavonoid biosynthesis. Jackson *et al.* (1995) identified a mutant altered in anthocyanin formation and *CHS* expression. This mutant, termed *icx1* (*i*ncreased *c*halcone synthase *ex*pression), was isolated by screening mutagenised transgenic seedlings for altered expression of GUS fused to

the promoter of the *Sinapsis alba 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. *icx1* has an elevated response to UV and blue light in *CHS* expression (Jenkins, 1997). 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 (Jackson *et al.*, 1995).

Thus, the *icx1* mutant appears to have increased sensitivity to light with respect to UV/blue light-regulated expression of genes involved in flavonoid biosynthesis. No alteration in *CAB* transcripts was observed, indicating that *icx1* is not altered in the expression of all light-regulated genes. An interpretation of this recessive mutation is that ICX1 encodes a negative regulator of *CHS* and other UV/blue light regulated genes.

The *icx1* mutant is also altered in several aspects of epidermal development, including altered leaf shape, a reduced number of leaf trichomes, aberrant root development, altered epidermal development and alterations to the seed coat (Jackson and Jenkins, 1995; Jenkins, 1997). It is therefore likely that ICX1 functions in the epidermis to control aspects of gene expression and development.

A second mutant isolated using the above screen, which is not allelic to *icx1*, was recently reported. This mutant, designated *icx2*, shows strongly elevated *CHS-GUS* expression, *CHS* transcript accumulation and anthocyanin induction. Unlike *icx1*, the mutant appears similar in morphology to wild-type in both light and dark grown conditions (Jenkins, 1997). Hence ICX2 defines a further negative regulator of *CHS* transcription.

1.4.2 *Arabidopsis* COP/DET/FUS mutants

Photomorphogenic mutants have been isolated which are altered in signal transduction events involved in photomorphogenesis, including the regulation of light-induced genes. Screening of *Arabidopsis* seeds in the dark led to the isolation of eleven mutants that undergo photomorphogenesis in the dark. Eight were called *co*nstitutive *p*hotomorphogenic (*cop*) mutants (Deng *et al.*, 1992; Deng, 1994) and three *de-et*iolated (*det*) mutants (Chory *et al.*, 1989; Chory, 1993). Meanwhile, screening for *Arabidopsis* mutants defective in embryogenesis resulted in the isolation of twelve mutants with a high accumulation of anthocyanins in cotyledons of immature embryos. These mutants were given the name *fusca*, (latin for dark purple). It was later determined that eight of the *fusca* mutants corresponded to severe *cop/det* alleles (Misera *et al.*, 1994). The remaining four mutants represented novel *fusca* loci. Ten of the *cop/det/fus* mutants displayed pleiotropic photomorphogenic features in the dark, including constitutive expression of light-regulated genes, whereas the remaining five mutants only displayed a subset of these phenotypes (Wei and Deng, 1996).

The recessive nature of these pleiotropic mutations implies that the gene products act as negative regulators, suppressing photomorphogenesis in the dark. Light has been proposed to inactivate the gene products, switching the plant from a skotomorphogenic program to a photomorphogenic proogram. This hypothesis is supported by over-expression of COP1 in wild-type *Arabidopsis* plants. Transgenic *Arabidopsis* plants over-expressing COP1 display partially etiolated characteristics in the light. The long hypocotyl phenotype of COP1 overexpressors is especially noticeable under far-red and blue light (McNellis *et al.*, 1994b).

Double mutants between several *det/cop* mutants and CRY1-deficient and phytochrome-deficient mutants have been produced. In each case the double mutant resembles the *det/cop* parent, indicating that the DET/COP components function downstream of phytochromes and CRY1 (Chory, 1992; Ang and Deng,

1994; Misera *et al.*, 1994; Wei *et al.*, 1994a; 1994b). However, in addition to light regulated characteristics, *det/cop* mutants are also altered in the tissue specific regulation of gene expression (Chory and Peto, 1990; Deng and Quail, 1992) and the transduction of a range of environmental and endogenous signals (Castle and Meinke, 1994).

1.4.3 Structure of the COP/DET/FUS proteins

To date, four of the pleiotropic DET/COP/FUS loci have been cloned. COP1 encodes a 74 kDa protein with a novel combination of recognizable domains: an N-terminal zinc-binding RING-finger, a predicted coiled coil, and C-terminal WD-40 repeats (Deng *et al.*, 1992; McNellis *et al.*, 1994a). COP1 shows sequence homology with the $TAF_{II}80$ subunitof the *Drosophila* transcription factor$TF_{II}D$ in all but the zinc finger domain. COP1 also has functional nuclear localisation signals (von Arnim and Deng, 1994).

DET1 has no homology to known proteins (Pepper *et al.*, 1994), is predicted to be hydrophilic and contains two regions similar to nuclear localization signals. A DET1-GUS construct fused to a CaMV 35S promoter was introduced into leaf protolasts and GUS staining showed that the DET1-GUS fusion was targeted to the nucleus (Pepper *et al.*, 1994). Levels of *DET1* mRNA were similar for both light- and dark-grown plants.

FUS6 (COP11) is a hydrophilic protein (Castle and Meinke, 1994). Unlike COP1 and DET1, no localisation signals were evident, suggesting that FUS6 is cytoplasmic. The predicted amino acid sequence showed no homology to any known proteins. It has no DNA binding motifs, or any homology to regulatory proteins. However, FUS6 does contain possible protein kinase C phosphorylation sites near the C-terminus and, in the N-terminal region, possible ATP/GTP binding sites, a possible glycosylation site and possible metal binding sites. FUS6

expression was detected in seedlings and mature plants, and present in leaves, flowers, immature siliques and light-grown roots. However, *FUS6* mRNA was not detected in etiolated seedlings, suggesting an important role prior to germination.

COP9 was predicted to be a hydrophilic protein (Wei *et al.*, 1994a), and like DET1 and FUS6 the peptide sequence was not significantly homologous to any previously identified protein sequences. Thus COP9 also appears to be a novel plant protein. However, there are two conserved putative phosphorylation sites, one a protein kinase C-dependent site and the other a cyclic AMP-dependent protein kinase phosphorylation site.

1.4.4 The COP9 complex is post-translationally regulated by light

Further analysis of the *cop9* mutant provided biochemical evidence that the COP9 protein is likely to function as part of a complex in the cytosol. Wei *et al.*, (1994a) reported that COP9 encodes a 22 kDa polypeptide which is present in large complexes of varying sizes in dark-grown *Arabidopsis* seedlings. The size of the COP9 complex, however, is light-regulated. Although COP9 is still present in a complex in illuminated seedlings, the larger size fraction was abolished or reduced. COP9 from illuminated tissue is present in a smaller 560 kDa complex.

Interestingly, although *COP9* mRNA accumulates at higher levels in *cop8* and *fus6* mutant seedlings than in the wild-type, no detectable COP9 protein is detectable in the mutants *cop8* and *fus6* (Wei *et al.*, 1994b). This implies that COP8 and FUS6 are either specifically involved in formation or stablity of the COP9 complex, or COP8 and FUS6 are components of the COP9 complex. Evidence was presented that the FUS6 and COP9 proteins co-purified from cauliflower heads (Chamovitz *et al.*, 1996). Further studies (Staub *et al.*, 1996),

found that accumulation of the COP9 and FUS6 proteins is co-ordinated throughout plant development, and antibodies to COP9 and FUS6 selectively co-immunoprecipitate both proteins from plant protein extracts. Like COP9, the FUS6 protein is only present in a large protein complex.

As previously shown for DET1, immunolocalization studies using cauliflower protoplasts (Chamovitz *et al.*, 1996) and *Arabidopsis* protoplasts (Staub *et al.*, 1996) clearly indicate that COP9 and FUS6 are localized in the nucleus. However, no direct interaction between COP9 and FUS6 has been demonstrated. The physical association has been proposed to be mediated via other unidentified components of the complex (Chamovitz and Deng, 1997), and clearly further studies are required to determine the nature of these interactions.

1.4.5 COP1 localization is regulated by light

Like COP9, light has no effect on mRNA and protein levels of COP1 (Deng *et al.*, 1992; McNellis *et al.*, 1994a). However, using a GUS-COP1 fusion, it was demonstrated that the nucleocytoplasmic partitioning of COP1 is regulated by light (von Arnim and Deng, 1994). In darkness, GUS-COP1 localizes in the nucleus. Transfer of the seedlings to light reduces COP1 abundance in the nucleus (Chamovitz *et al.*, 1996; von Arnim *et al.*, 1997). The structure of COP1 suggests that it may be involved in suppressing gene expression either directly or by associating with the general transcriptional machinery (Torii and Deng, 1997).

COP1 does not localize to the nucleus in the dark in all mutants known to lack the COP9 complex (Chamovitz *et al.*, 1996). It has been proposed that the COP9 complex interacts with COP1 in the dark but not in the light, and it may be that the COP9 complex functions by binding COP1 in the dark. A light signal could result in the release of COP1 from the complex and its export to the cytoplasm, leaving the 560 kDa COP9 complex (Chamovitz and Deng, 1997). It

will be interesting to see if a physical association between COP1 and the COP9 complex in the dark is demonstrated.

1.5 Conclusions

Considerable advances have been made recently in UV/blue light perception and signal transduction in higher plants. Undoubtedly a genetic approach has been invaluable in the isolation and characterisation of the CRY1 photoreceptor and the photoreceptor for phototropism. This approach can be used to isolate and clone new UV/blue and UV-B photoreceptors. Additionally, pharmacological, biochemical and electrophysiological experiments have enabled the characterisation of UV/blue light signal transduction components. However, there is currently little known about UV/blue light regulated components regulating gene expression in higher plants. It will be a priority now to examine how UV/blue light photoreceptors initiate signal transduction pathways regulating gene expression, and how these events interact with the ICX and COP/DET/FUS components.

1.6 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 in higher plants. A pharmacological approach was utilised to identify signal transduction components. The UV-A/blue light regulation of *rbcS* gene expression in *Phaseolus vulgaris* protoplasts was studied initially, although this system proved to be rather difficult. Hence the research was extended to the UV-A/blue and UV-B light regulation of

the *Arabidopsis CHS* and *PAL* genes in an *Arabidopsis* photomixotrophic cell culture. The *Arabidopsis* cell culture was a particularly attractive system for several reasons. Initial information on the UV/blue light signal transduction components involved in *CHS* expression had already been obtained and a number of *Arabidopsis* mutants, including the *hy4* mutant which is deficient in the CRY1 photoreceptor, were available.

The important questions which were addressed in this study were: (i) What are the UV/blue light signal transduction components regulating *rbcS* expression in *P.vulgaris* protoplasts? (ii) Are these components similar to those involved in the UV-A/blue light regulation of *CHS* and *PAL* in *Arabidopsis* cells? (iii) Is plasma membrane electron transport involved in the UV-A/blue and UV-B light regulation of *CHS* and *PAL* gene expression? (iv) What is the role of calcium in UV-A/blue and UV-B light signal transduction regulating *CHS* and *PAL* expression? (v) Are the UV-B and UV-A/blue light phototransduction pathways identified in this study the same or distinct?

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

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

2.1.2 Restriction endonucleases

All restriction endonucleases used in this study were supplied from Amersham International (UK) together with their reaction buffers, which were provided at a 10 x concentration.

2.1.3 Plasmids and bacterial strains

The plasmids used in this study are described in Table 2.1. *E.coli* strain DH5α was used as the recipient bacteria to enable amplification of all plasmids for small scale plasmid DNA preparations. *Agrobacterium* strain, GV3101, was used for *Arabidopsis* transformation with pMAQ2.

2.1.4 Antibiotics

Antibiotics were supplied by Sigma Chemical Co (UK). Ampicillin was made as a stock solution (25 mg ml^{-1}) in dH_2O and used at a working concentration of 50 μg ml^{-1}. Tetracycline was made as a stock solution (5 mg ml^{-1}) in EtOH and used at a working concentration of 50 μg ml^{-1}. Gentamycin was made up as a stock colution of (10 mg ml^{-1}) in dH_2O and used at a working concentration of 25 μg ml^{-1}.

Figure 2.1 Plasmid DNA used in this study.

Plasmid DNA and their source, used in this study are described in the table.

Plasmid	Description	Reference
pPvSS191	*Phaseolus vulgaris rbcS 3* in pUC8	Knight and Jenkins (1992)
pH1	*Phaseolus vulgaris H1* in pAT153	Lawton and Lamb (1987)
pCHS	*Arabidopsis CHS* in pUC19	Trezzini *et al.* (1993)
pPAL	*Arabidopsis PAL* in pUC19	Trezzini *et al.* (1993)
pTCH3	*Arabidopsis TCH3* in pBluescript	Braam (1992)
pPR1	*Arabidopsis PR-1* in pBluescript	Uknes *et al* (1992)
pMAQ2	cauliflower mosaic virus 35S: *Aequoria victoria* apoaequorin:cauliflower mosaic virus 3' gene construct in pBIN19	Knight *et al.* (1991)

Kanamycin was made as a stock solution (50 mg ml^{-1}) in dH_2O and used at a working concentration of 50 $\mu g\ ml^{-1}$. Ampicillin and kanamycin stocks were filter sterilized through a Nalgene filter. Antibiotics were stored at -20 °C.

2.1.5 Radiochemicals

[α-^{32}P] dCTP was supplied by Amersham.

2.2 General preparatory 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 solution was measured using Whatman Narrow Range pH paper (ph 6.0-8.0) (Whatman, Maidstone, UK).

2.2.2 Autoclaving

Equipment and solutions were sterilized at 15 psi for 20 mins in a Laboratory Thermal Equipment Autoclave 225E.

2.2.3 Filter sterilization

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

2.2.4 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 plasticware was used throughout.

2.3 Preparation of plant material

2.3.1 Growth of *Phaseolus vulgaris*

Phaseolus vulgaris cv Tendergreen seeds (Sharps International, Sleaford) were planted, 10-12 to a pot, in moist potting compost (ICI Agrochemicals, Farnham) and germinated under continuous white light (100 μmol m^{-2} s^{-1}) at a temperature of 20°C in a controlled environment room until primary leaves were fully expanded. Plants were then transferred to a dark cabinet in a controlled environment dark growth chamber for 2 days (dark-adapted plants). All further manipulations of plant material were carried out under a green safety light.

2.3.2 *Phaseolus vulgaris* protoplast isolation

This method was obtained from Dr. N.A.R. Urwin (Division of Biochemistry and Molecular Biology, University of Glasgow). 0.8% (w/v) cellulase R10 and 0.25% (w/v) macerozyme R10 (Yakult Honsha Co Ltd., Japan) enzyme solution was made up in 0.4 M mannitol (Sigma) in CPW salts (272 mg l^{-1} potassium dihydrogen orthophosphate, 101 mg l^{-1} potassium nitrate, 1480 mg l^{-1} calcium chloride, 246 mg l^{-1} magnesium sulphate, 0.16 mg l^{-1} potassium iodide and 0.025 mg l^{-1} copper sulphate). The enzyme solution was filter sterilized through a 0.2 μM filter (Nalgae) and kept on ice until used.

Primary leaves were removed from dark-adapted *Phaseolus vulgaris* plants under green safety light. Alumina powder (Sigma, type A-5) was sprinkled on the

upper surface of each leaf so that the leaf surface was covered, and the alumina rubbed gently across the surface of the leaves with a gloved finger. The leaves were washed gently in tap water several times to remove the alumina powder and 2-3 leaves placed upper surface down in a 90 mm Petri dish containing 25 ml enzyme solution. Digestion was allowed to proceed overnight in darkness at room temperature.

Protoplasts were isolated from the leaves by gently swirling the Petri dish. The contents of the Petri dish were then filtered through muslin and a 85 ml screen cup with a 70 - 80 µm steel mesh (Sigma). The filtered solution was gently tipped into 50 ml sterile centrifuge tubes. Protoplasts were returned to a dark cabinet and pelleted by allowing the solution to stand on ice for an hour. The supernatant was gently removed and protoplasts were washed by resuspending in 1/2 the original volume with culture buffer. Protoplasts were pelleted on ice for an hour as above, and the supernatant removed. The protoplast suspension was diluted with protoplast culture medium to give approximately 1 x 10^6 protoplasts per ml in a 10 ml centrifuge tube. Cells were then treated for the times indicated in Chapter 3.

2.3.3 Protoplast cell count and viability

Viability of protoplasts 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. 200 µl of this solution was mixed with an equal volume of protoplast suspension and left at room temperature for 2 mins. Protoplasts were viewed using a fluorescence microscope (using blue illumination). Viable protoplasts stained green whereas non-viable protoplasts appeared red (due to chlorophyll fluorescence). Percentage viability in a random field of view could be determined as (number of viable protoplasts/total number of protoplasts) x 100. Viability was determined as mentioned in Results section.

Protoplasts were counted using a haemocytometer. A sample of the protoplast suspension was introduced beneath the cover slip. The number of protoplasts enclosing the triple-lined square in the centre were determined by counting the number of protoplasts in 1 row of the centre square (n) including those that touched the top and left edges. The number of protoplasts per ml was calculated as $5n \times 10^3$.

2.3.4 *Arabidopsis thaliana* cell culture growth

The *Arabidopsis* cell culture, originally described in May and Leaver (1993), was supplied by Dr J.M. Christie (Division of Biochemistry and Molecular Biology, University of Glasgow). 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 supplied by Sigma), 3% (w/v) sucrose, pH 5.8). Suspension cultures were grown at 20°C under continuous low fluence rate white light (20 μmol m^{-2} s^{-1}) with constant shaking at 110 rpm. Cells were subcultured every 7 days by transferring 20 ml of culture to 180 ml fresh sterile culture medium.

For experimental treatments, on the third day after subculturing 10 ml cell aliquots were transferred to 50 ml tissue culture flasks (Nunclon™). Cells were treated, with constant shaking (80 rpm) for the times indicated in the Results.

2.3.5 Growth of *Arabidopsis thaliana* plants

Arabidopsis thaliana cv Landsberg *erecta* and *Arabidopsis hy4-2.23N* mutant seeds were supplied by the *Arabidopsis* Stock Centre (Nottingham, UK). *Arabidopsis* seeds were sown onto trays containing moist ICI potting compost, covered in clingfilm and placed at 4°C for 2 days to synchronize germination.

Plants were then grown in white light (20 μmol m^{-2} s^{-1}) at 20°C in controlled environment growth rooms.

2.4 Treatment of plant material

2.4.1 Light treatments used

Illuminations were carried out in controlled environment rooms at 20°C. The spectra of all 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, Munchen, Germany), UV-A/blue light was supplied from Sylvania F36W fluorescent tubes, (GE Lighting), red light was supplied from covering the white fluorescent tubes in Deep Golden Amber, (LEE filters). UV-B was supplied by Philips TL 40W/12 ultra-violet fluorescent tubes covered with cellulose acetate, changed every 24 hours, to remove UV-C wavelengths. In order to determine that spikes in the UV-B spectra occuring above 320 nm (Figure 2.2 D) are not inducing gene expression, Christie and Jenkins (1996) used a filter to remove wavelengths below 320 nm, and an equal fluence of this light treatment did not induce *CHS* expression in *Arabidopsis* cells.

The fluence rates of the light regimes used are described for each experiment and were obtained by varying the number of tubes in the growth area and distance of the plant material from the light source.

2.4.2 Fluence rate measurement

The photon fluence rates (400-700 nm)of the visible light sources were measured using a Li-Cor quantum sensor, model Li-185B. Photon fluence rates of the UV-containing light sources were measured using a spectroradiometer (Macam SR9910).

Figure 2.2 Light treatments 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.

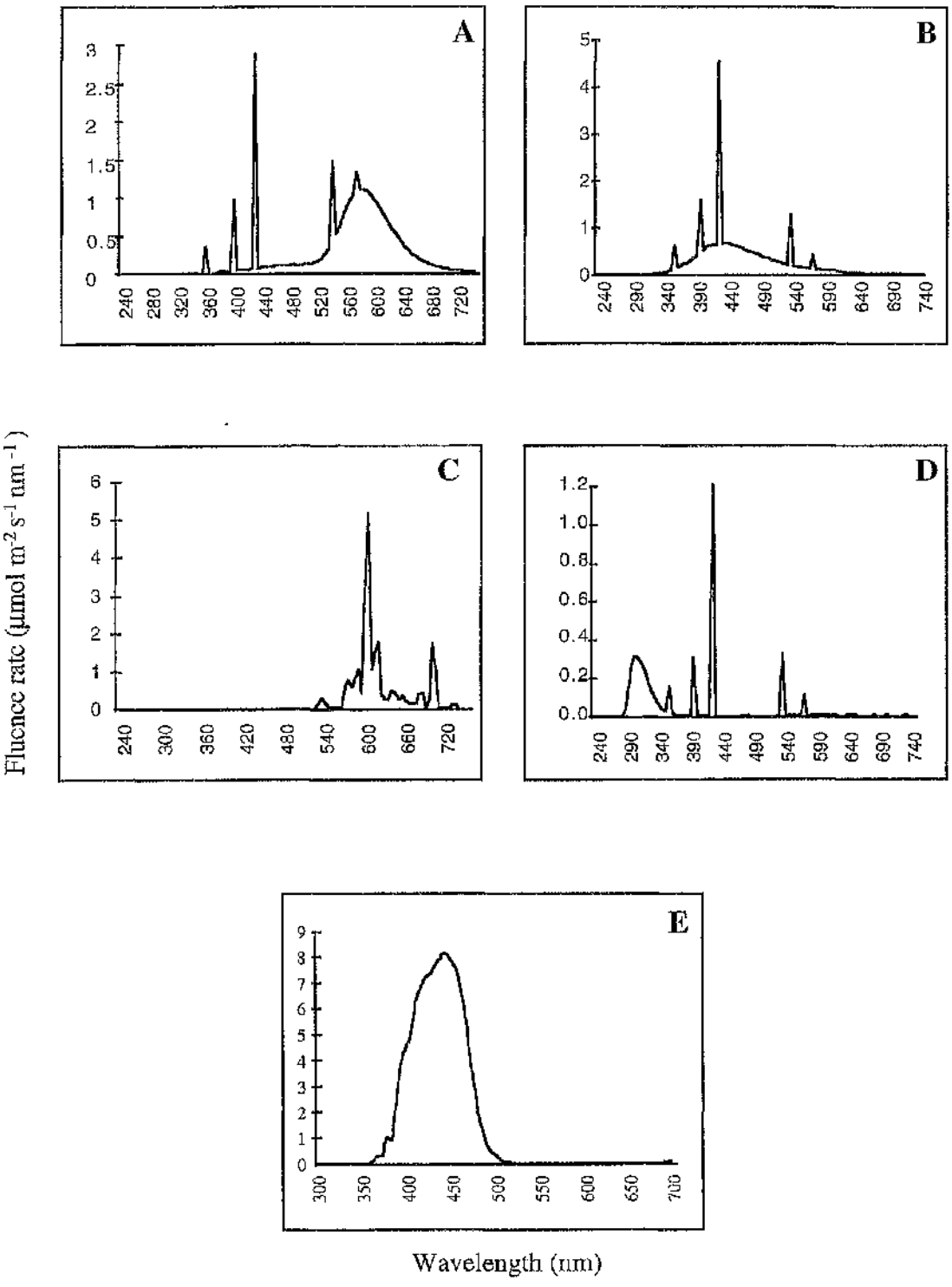
(A) White (100 μmol m^{-2} s^{-1})

(B) UV-A/blue (80 μmol m^{-2} s^{-1})

(C) Red (80 μmol m^{-2} s^{-1})

(D) UV-B (3 μmol m^{-2} s^{-1})

(E) UV-A/blue (used for aequorin measurements) (650 μmol m^{-2} s^{-1})


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

2.4.3 Chemical agonists/antagonists used

All chemical agonists/antagonists except cantharidin, staurosporine, genistein, hydrogen peroxide (H_2O_2), erythrosin-B and A23187, were obtained from Sigma. H_2O_2 was supplied by Analytical Reagents (Dorset, UK), erythrosin-B from Aldrich (Dorset, UK). Cantharidin, staurosporine, genistein and A23187 were from Calbiochem-Novabiochem (Nottingham, UK).

Diphenylene iodonium (DPI), nifedipine, N-(6-aminohexyl) 5-chloro-1-naphthalene sulphonamide (W-7), genistein, cantharidin, staurosporine and A23187 were dissolved in DMSO to yield a 50 mM (DPI), 10 mM (nifedipine and W-7), 100 mM (genistein and cantharidin) or 1 mM (staurosporine, A23187) stock. Ruthenium red, verapamil, lanthanum chloride, cycloheximide, N-acetyl-L-cysteine (NAC), pyrrolidinedithiocarbamate (PDTC), reduced glutathione (GSH), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 3-amino-1,2,4-triazole (ATZ), L-butathione-[S,R]-sulfoximine (BSO), salicylic acid (SA), potassium ferricyanide ($K_3Fe(CN)_6$) and dichlorophenol-indophenol (DCPIP) were dissolved in dH_2O at 100 mM (NAC, PDTC, EGTA, GSH and $K_3Fe(CN)_6$), 10 mM (ruthenium red, verapamil, lanthanum chloride, cycloheximide, BSO and DCPIP), 50 mM (SA) or 1 mM (erythrosin-B). H_2O_2 was supplied as a 30% (w/v) solution in H_2O.

Chemicals were added to protoplasts and cells in at the required concentration indicated in the Results, so that the volume of chemical added did not exceed 100 µl in 10 ml.

2.5 Amplification of plasmid DNA

2.5.1 Preparation of competent cells

A single colony of *E.coli* was grown overnight in 10 ml of ψ medium (2% (w/v) bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 0.4% (w/v) $MgSO_4$, 10 mM KCl) at 37°C with constant shaking. The following day 500 ml of ψ broth in a 2 L conical flask was inoculated with the overnight culature and shaken at 37°C until OD at 550 nm reached 0.35. Cells were pelleted at 2,000 *g* for 5 mins at 4°C and the supernatant removed. Cells were resuspended in 75 ml of TFB1 (100 mM RbCl, 50 mM $MnCL_2$, 30 mM KOAc, 10 mM $CaCl_2$, 15% (v/v) glycerol, pH 5.8 with glacial acetic acid) and incubated on ice for 60 mins. Cells were centrifuged as before, and resuspended in 20 ml of TFB2 (10 mM MOPS (Sigma), 10 mM RbCl, 75 mM $CaCl_2$, 15% (v/v) glycerol, pH 7.0). 200 µl aliquots in 1.5 ml Eppendorf® tubes were frozen in liquid nitrogen and stored at -80°C.

2.5.2 Transformation of competent cells

Frozen competent cells, prepared as in 2.5.1, were thawed and kept on ice. Approximately 200 ng of plasmid DNA, in a volume of 1-5 µl was added to the cells, and the contents mixed gently, before returning to ice for 20 mins. Cells were heat shocked by placing in a 37°C water bath for 30 secs, then returned to ice for 2 mins. 200 µl of ψ broth was added to each tube, and cells were incubated at 37°C for 50 mins with constant shaking to allow expression of antibiotic resistance genes. Cells were pelleted for 30 secs at 10,000 *g* and resuspended in 100 µl of ψ broth. Cells were plated on LB agar (1% (w/v) bacto-tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% (w/v) agar) plates in 90 mm Petri dishes with kanamycin.

2.5.3 Small scale plasmid preps

Plasmid preps were carried out using the Wizard™ Plus Minipreps DNA Purification System (Promega) in accordance with the manufacturer's instructions. 5 ml of LB medium, supplemented with kanamycin, was inoculated with a single bacterial colony. The culture was grown at 37°C with constant shaking overnight. Cells were pelleted at 10,000 *g* for 10 mins. The supernatant was decanted, and pelleted cells resuspended in 300 µl of Wizard™ cell resuspension solution which was transferred to a 1.5 ml Eppendorf® tube. 300 µl of Wizard™ cell lysis solution was added and the contents mixed by inverting 4 times. 300 µl of Wizard™ neutralization solution was added, the contents mixed by inverting several times, and centrifuged at 10,000 *g* for 5 mins.

Meanwhile, a Wizard™ microcolumn/syringe assembly was attached to a vacuum manifold (Promega), and the stopcock closed. 1 ml of Wizard™ DNA purification resin was pipetted into the barrel of the minicolumn/syringe assembly and a vacuum applied. The supernatant from the previous step was pipetted into the microcolumn/syringe assemby containing the resin. The stopcock was opened and a vacuum applied until all the resin/lysate mix had passed through the column, and the vacuum broken. 2 ml of Wizard™ column wash solution was added to the syringe barrel, and again a vacuum applied. The syringe was then discarded and the minicolumn transferred to a 1.5 ml Eppendorf® tube and centrifuged at 10,000 *g* for 2 mins. The minicolumn was transferred to a fresh 1.5 ml Eppendorf® tube, and 50 µl of TE buffer (10 mM Tris, 1 mM Na_2EDTA, pH 8) added, before centrifugation again at 10,000 *g* for 20 secs to elute the plasmid DNA. The minicolumn was discarded and plasmid DNA stored at -20°C.

2.6 Isolation of total RNA from plant material

2.6.1 Preparation of phenol

Five hundred grams of phenol (detached crystals) was melted at 65°C and 0.58 g of 8-hydroxyquinoline added before the phenol had cooled. The solution was saturated with 100 mM Tris-HCl (pH 8) amd mixed for 10 mins. After allowing 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) 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.

2.6.2 Isolation of total RNA from *P.vulgaris* protoplasts

Protoplasts were immediately placed on ice. The protoplasts were centrifuged for 5 mins at 150 *g*, the supernatant was removed and 0.6 ml of extraction buffer (5 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sarcosyl, 2 mM EDTA, 1 mM ß-mercaptoethanol (BDH, Poole, Dorset) and 50 mM Tris-Cl, pH 7.6) added. The tubes were vortexed for 20 seconds and the contents transferred to sterile 2 ml Eppendorf® tubes. 0.6 ml of phenol and 0.6 ml chloroform: isoamyl alcohol (24:1 v/v) were added and the tubes vortexed and returned to ice. The samples were centrifuged at 10,000 *g* for 10 mins at 4°C. The upper aqueous phase was transferred to a fresh 1.5 ml Eppendorf® tube which contained 1 ml of ice-cold EtOH and 0.4 ml ice-cold 3 M Na acetate pH 5.5, and nucleic acids precipitated at -20°C for 1 hour.

Each tube was centrifuged at 10,000 *g* for 10 mins at 4°C and the pellet was washed in 200 µl of ice-cold 70% EtOH and then centrifuged at 10,000 *g* for 5 mins. The pellet was vacuum dried for 10 mins and resuspended in 30 µl of DEPC-treated sterile dH_2O. RNA samples were stored at -80°C.

2.6.3 Isolation of total RNA from the *Arabidopsis* cell culture

A 2 ml aliquot of the *Arabidopsis* cell culture was transferred to a sterile 2 ml Eppendorf® tube and centrifuged at 10,000 *g* for 10 mins. The supernatant was carefully removed by aspiration and the pellet immediately frozen in liquid nitrogen. Frozen cell tissue was then ground to a fine powder using a mortar and pestle, and transferred to a fresh 2 ml Eppendorf® tube containing 0.4 ml of extraction buffer (as above) and vortexed for 10 secs. 0.4 ml each of phenol and chloroform were added, and the tube vortexed for 20 secs before centrifuging for 10 mins at 10,000 *g* at 4°C. The upper layer was removed to a fresh Eppendorf® tube and 2 volumes of EtOH added. The contents were mixed by inverting several times and nucleic acids precipitated at - 20°C for 1 hour.

After precipitation, the tube was centrifuged at 4°C for 10 mins at 10,000 *g* and the supernatant discarded. The pellet was resuspended with 0.2 ml of 3 M Na acetate (pH 5.5). The tube was centrifuged as before for 5 mins and the pellet washed in 70% EtOH and centrifuged as before for 10 mins. The pellet was dried and dissolved in DEPC-treated dH_2O. RNA samples were stored at -80°C.

2.6.4 Isolation of total RNA from *Arabidopsis* plant material

Total RNA from *Arabidopsis* plant tissue was isolated using the PURE*script* ™ RNA Isolation Kit, according to the manufacturer's instructions. 5-10 mg of tissue was harvested and immediately frozen in liquid nitrogen. Frozen tissue was ground to a fine powder using a mortar and pestle, and transferred to a fresh 1.5 ml Eppendorf® tube containing 300 µl PURE*script* ™ cell lysis solution and vortexed for 20 secs. 100 µl of PURE*script* ™ protein-DNA precipitation solution was added to the cell lysate, and the Eppendorf® tube inverted gently a few times, and placed on ice for 5 min.

The tube was centrifuged for 3 mins at 10,000 *g* and the supernatant removed to a fresh sterile 1.5 ml Eppendorf® tube. 300 µl of isopropanol was added, the Eppendorf® tube inverted gently a few times and centrifuged as above for 3 mins. The supernatant was decanted off, and pelleted RNA washed in 70% EtOH, and centrifuged as above for 1 min. The pellet was dried and dissolved in DEPC-treated dH_2O. RNA samples were stored at -80°C.

2.7 Isolation of genomic DNA from *Arabidopsis* plants

For each sample, 0.1-0.2 g of whole plant tissue was frozen in liquid nitrogen and stored at -80°C. Tissue was ground to a fine powder with liquid nitrogen in a mortar and pestle, and transferred to a centrifuge tube containing 15 ml of extraction buffer (1.3% (w/v) SDS, 100 mM Tris pH 8, 50 mM EDTA pH 8, 500 mM NaCl) and vortexed for 20 secs. The tube was transferred to a 65°C water-bath for 1 hour and subsequently was cooled on ice. 5 ml of 5 M ammonium acetate was added and the tube shaken to mix the contents, then centrifuged for 20 mins at 1,500 *g*.

The supernatant was poured into 14 ml isopropanol in a fresh centrifuge tube, and mixed by shaking and centrifuged for 20 mins as before. Supernatant was discarded and the pellet washed in 10 ml of 70% EtOH and again centrifuged for 20 mins. The pellet was air-dried for 10-15 mins, resuspended in 500 µl sterile dH_2O, transferred to a 1.5 ml Eppendorf® tube and stored at 4°C overnight.

200 $\mu g ml^{-1}$ RNAse was added to the resuspended DNA, and the Eppendorf® tube incubated at 37°C for 1 hour. The Eppendorf® tube was then transferred to a 65°C water-bath for 10 mins, centrifuged for 10 mins at 10,000 *g*, dissolved DNA transferred to a fresh Eppendorf® tube and the pellet discarded. 2.5 volumes of EtOH and 0.1 volume of 5 M NaCl was added to the sample and transferred to -80°C for 1 hour.

The Eppendorf® tube was centrifuged for 10 mins at 10,000 *g*, supernatant discarded, pellet washed in 70% EtOH, and re-centrifuged for 10 mins as before. Pelleted DNA was air-dried for 10-15 mins, and resuspended in 100 µl of sterile dH_2O. DNA was stored at 4°C.

2.8 Manipulation of DNA and RNA

2.8.1 Quantification of DNA and RNA

An aliquot of the nucleic acid solution was diluted to 1 ml with dH_2O and the absorbance measured between 220 nm and 320 nm against a dH_2O blank. An absorbance at 260 nm of 1.0 was taken to indicate the following concentrations:

Form of nucleic acid	Concentration (µg ml^{-1})
Double stranded DNA	50
Single stranded DNA and RNA	38

2.8.2 Digestion of DNA with restriction endonucleases

The DNA to be restricted was prepared in a solution of 1 x the appropriate buffer and 1-20 units of restriction enzyme(s) was added ensuring that its concentration did not exceed 10% (v/v). The reaction was incubated at 37°C for 2-24 hrs.

2.8.3 Denaturing electrophoresis of RNA

This method was used for gels that were to be blotted for hybridisation analysis. 1.95 g of agarose (Gibco BRL) was melted in 109 ml of DEPC-treated dH_2O and allowed to cool to 60°C. 15 ml of 10 x MOPS (0.2 M MOPS (Sigma Co.), 50 mM Na acetate, 10 mM EDTA, pH 7.0) and 25.8 ml of formaldehyde (30% (v/v)

Sigma) was added, the solution gently swirled and poured into a gel tray and allowed to set. After the gel was set, the gel tray was placed in the electrophoresis apparatus and submerged in 1 x MOPS buffer.

5-20 μg of RNA 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% (v/v) formaldehyde in a volume no greater than 50 μl. This solution was heated to 65°C for 5 mins and placed on ice. One-tenth volume of loading buffer solution (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol (both supplied by BDH), 25% (w/v) Ficoll 400 (Pharmacia, Milton Keynes)) was added. The RNA sample was then loaded onto the gel with an automatic 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 visualised under UV light (Spectroline® transilluminator, Model TC-312A).

2.8.4 Electrophoresis of DNA

The appropriate concentration of agarose (0.6-1.0% (w/v)) was added to the volume of 1 x TBE (0.09 M Tris-borate, 2 mM Na_2EDTA) required for the gel being cast. The agarose suspension was melted and allowed to cool to 60°C at which point 10 mg ml^{-1} EtBr was added to a final concentration of 1 μg ml^{-1}. The agarose solution was poured into a gel tray and allowed to set. After the gel was set, the gel tray was placed in the electrophoresis apparatus and submerged in 1 x TBE buffer. The DNA samples were mixed with one-tenth loading buffer solution and loaded with an automatic 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.

2.8.5 Isolation of DNA fragments from agarose gels

The DNA fragment was separated from residual DNA by agarose gel electrophoresis as described in Section 2.8.4 and identified by EtBr staining under UV light. The DNA fragment was cut out of the gel with a clean scalpel blade and transferred to a 1.5 ml Eppendorf® tube The weight of the gel slice was determined, and the fragment cut into small pieces and divided between separate Eppendorf® tubes so that each Eppendorf® tube contained no more than 0.1 g of gel. 500 µl of phenol was added to the Eppendorf® tube and the mixture vortexed until the gel fragments had emulsified. The tube was then frozen in liquid nitrogen and immediately centrifuged at 10,000 *g* for 10 mins. The upper phase was 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. The upper phase was transferred to a fresh Eppendorf® tube, an equal volume of chloroform added, the mixture vortexed and the centrifugation step repeated. The upper phase was transferred to a fresh Eppendorf® tube and 2 volumes of EtOH added along with one-tenth volume 3 M Na acetate (pH 5.5). DNA was precipitated at -20°C for at least one hour. The Eppendorf® tube was centrifuged at 10,000 g for 5 mins. 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 mins and then resuspended in an appropriate volume of sterile dH_2O.

2.9 Radiolabelling of DNA

2.9.1 Preparation of probe DNA from plasmid DNA

The DNA sequence to be labelled was cut out of the host plasmid using appropriate restriction enzyme(s) and DNA purified as described in Section 2.8.5. The amount of DNA was estimated by running an aliquot of the fragment on an agarose gel as described in Section 2.8.4 and comparing it to known amounts of DNA loaded onto the same gel.

2.9.2 PCR amplification of *GST* probe DNA

PCR of the GST cDNA used *Arabidopsis* genomic DNA prepared as described in Section 2.7. Reagents were added to a 0.5 ml centrifuge tube in the following order; 5 µl *Taq* buffer (Promega), 8 µl 10 x dNTP, 1 µl of each primer donated by Dr. M.R.Knight (Oxford University, UK), 5 µl $MgCl_2$, 5 µl *Taq* polymerase (Promega, 2.5 $U.\mu l^{-1}$), 1 µl genomic DNA and H_2O to 50 µl. All the reagents were mixed thoroughly, layered with two drops of mineral oil (Sigma) to reduce evaporation and the tubes placed in a thermal cycler (Perkin-Elmer 2400, Perkin-Elmer, Cetus, USA).

The thermal cycler was programmed to denature the sample for 5 min at 94°C and then to complete 30 cycles of 94 °C for 1 min (denaturation), 60 °C for 1 min (primer annealing) and 72°C for 1 min (extension). At the end of the last cycle the sample was heated for a further 15 min to ensure full extension of the product and then cooled down to 4°C.

2.9.3 Radiolabelling of probe DNA

DNA was labelled with [α–^{32}P]dCTP using the Rediprime DNA labelling kit (Amersham) according to the manufacturer's instructions. 25-50 ng of DNA to be

labelled was made up to a volume of 45 µl in a sterile 1.5 ml Eppendorf® tube. DNA was denatured in a boiling water bath for 5 mins. The tube was spun briefly, denatured DNA added to the labelling mix (dATP, dGTP, dTTP, exonuclease-free 'Klenow' enzyme and random nonamer primers), and the contents mixed by gently flicking the tube. After a brief centrifugation, 50 µCi of [α-^{32}P]dCTP was added and the reaction mix incubated at 37°C for 10-15 mins.

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

A 0.5 ml Eppendorf® tube was placed inside a 1.5 ml Eppendorf® tube. A small hole was punched in the bottom of both tubes prior to this using a syringe needle. 50 µl of sterile glass beads were aliquoted into the small Eppendorf® tube, and 1 ml of Sephadex G-50 (Pharmacia) medium added. The Eppendorf® tube was spun for 4 mins at 700 *g* in a 10 ml centrifuge tube at room temperature. Another 500 µl of Sephadex was added and the tube spun as above for another 4 mins. 0.1 ml of TE buffer was added to the column and re-centrifuged as before.

The 0.5 ml Eppendorf® was removed from the 1.5 ml Eppendorf® and placed into a clean screw-top Eppendorf®, which was used to collect the eluate. After preparation of the radiolabelled cDNA probe as described in 2.9.3. The radiolabelled probe was added to the column, which was spun as before. The DNA solution in the Eppendorf® tube was stored at -20°C until used. Radiolabelled DNA was boiled for 5 mins in a boiling water bath immediately prior to use, and kept on ice.

2.9.5 Measurement of incorporation

Measurement 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 500 µl dH_2O. These were 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 centrifugation steps: (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 deoxynucleotide, template DNA and percentage incorporation of the radiolabel are known. A Rediprime reaction gave an incorporation of 50-80%. A 60% incorporation yielded a specific activity of 1.7×10^9 cpm μg^{-1}.

2.10 Hybridisation analyses of northern blots

2.10.1 Northern blotting

Denaturing RNA gel electrophoresis, described in 2.8.3, was used to separate RNA. A glass support plate was covered with a wick of Whatman 3MM paper. The wick was pre-soaked in 20 x SSC (3 M NaCl, 0.3 M *tri*-sodium citrate) and the ends of the wick were also dipped in a reservoir of 20 x SSC. The gel was placed on this support and wick. A piece of nylon membrane (Hybond-N, Amersham International), cut to the exact same size as the gel was placed onto the gel. Pieces of clingfilm were used to surround the gel. Three pieces of Whatman 3MM filter paper, also cut to the same size as the gel, were soaked in 2 x SSC and placed on top of the membrane. Stacks of thick tissue paper and a 500 g weight were placed on top and the blot left overnight.

The nylon membrane was carefully removed from the gel, marking on well positions, and baked at 80°C for two hours to fix the RNA to the membrane..

2.10.2 Pre-hybridisation and hybridisation of RNA

Northern blots were analysed using the method of Church and Gilbert (1984). The membrane, prepared as in Section 2.10.1, was pre-hybridised in 20 ml of pre-hybridisation solution (10 mg ml^{-1} BSA, 0.5 M Na_2HPO_4 (pH 7.2), 7% (w/v) SDS) in a plastic sealed bag at 55°C in a shaking water bath for 1-2 hours. The denatured cDNA probe, prepared as described in Section 2.9.4, was added using an automatic pipette and the bag sealed again. The hybridisation was carried out under the same conditions for 16-24 hours.

2.10.3 Washing conditions

After hybridisation, the membrane was washed initially in an appropriate volume of 2 x SSC, 1% (w/v) SDS for 10 mins at 55°C in a shaking water bath. This was followed by 1 x SSC, 1% (w/v) SDS for 5 mins at 55°C. The filter was washed at increasing stringency for 10-20 mins depending on the amount of radioactivity bound to the membrane.

2.10.4 Autoradiography

After washing, filters to be autoradiographed were sealed in a plastic bag and exposed to Fuji X-ray film (type RX) in a film cassette at -80°C for the appropriate length of time.

2.10.5 Stripping filters of bound radiolabelled probes and blocking agents

Bound probe and blocking agents were removed by pouring boiling 0.1% SDS onto the filters. The solution was allowed to cool to room temperature. This step was repeated 2-4 times.

2.11 Production of transgenic plants

2.11.1 *Agrobacterium* transformation

Agrobacterium strain GV3101 (Koncz and Schell, 1986) was incubated at 28°C with shaking overnight in 5 ml of YEP medium (1% (w/v) bacto-tryptone, 1% (w/v) bactoyeast, 0.5% (w/v) NaCl). 2 ml of the overnight culture was added to 50 ml of YEP and antibiotic. The culture was grown at 28°C in a shaker until the OD_{600} reached 0.72. The culture was chilled on ice and centrifuged at 3,000 *g* for 5 mins at 4°C. The supernatant was discarded and pelleted cells resuspended in 1 ml of ice-cold 20 mM $CaCl_2$. 100 µl aliquots of the cell suspension were transferred to 1.5 ml Eppendorf® tubes. 5 µg of plasmid pMAQ2 (containing the cauliflower mosaic virus 35S:apoaequorin:cauliflower mosaic virus 3' gene construct) donated by Dr. M.R. Knight (Oxford University, UK) was added to 100 µl of aliquoted cells and the tube immediately frozen in liquid nitrogen. Cells were thawed by incubating at 37°C in a waterbath for 5 mins. 1 ml of sterile YEP medium was added and the tube incubated at 28°C for 2 hours to allow expression of antibiotic resistance genes. The tube was centrifuged for 30 secs at 10,000 *g* and the supernatant discarded. Cells were resuspended in 100 µl of YEP medium and plated out onto YEP agar containing kanamycin and gentamycin. Plates were incubated at 28°C for 2 days.

5 ml of YEP medium containing kanamycin and gentamycin was inoculated with a single colony and grown overnight at 28°C in a shaker. 800 µl of the

culture was aliquoted into separate Eppendorf® tubes and 200 µl of glycerol added to each aliquot. Tubes were vortexed to mix and tubes immediately frozen in liquid nitrogen. Tubes were stored at -80°C.

2.11.2 Preparation of plant material

Arabidopsis thaliana Landsberg *erecta* and *A. thaliana* L*er hy4 2.23N* mutant seed was sown onto moist sterile compost in pots and transferred to 4°C for 2 days. Plants were germinated in continuous white light (100 µmol m^{-2} s^{-1}) at 20°C. 7 days later seedlings were thinned out to 6-8 plants per pot. The primary bolts were clipped off when they were 4-6 cm long to encourage growth of multiple secondary bolts. Infiltration was carried out 14 days later.

2.11.3 Preparation of infiltration medium

25 ml of YEP media containing kanamycin and gentamycin was inoculated with a stab of frozen glycerol stock of transformed *Agrobacterium* prepared as described in 2.11.1. The culture was grown overnight at 28°C with shaking. The following day, a fresh 400 ml of YEP with kanamycin and gentamycin was inoculated with the 25 ml overnight culture. The culture was grown at 28°C in a shaker until the OD_{600} was 2.0. The culture was centrifuged at 4,000 *g* for 15 mins. Cells were resuspended in 3 volumes of infiltration medium (1/2 MS salts, 1 x B5 vitamins, 5% sucrose, 10 µg l^{-1} BPA).

2.11.4 Vacuum infiltration of *Arabidopsis* plants

A glass beaker was filled with infiltration medium prepared as described in 2.11.3 and placed in a large bell jar. The pot containing 6-8 *Arabidopsis* plants, prepared as described in 2.11.2, was inverted and placed in the beaker so that all of the

flowers, stems and leaves were submerged in the infiltration medium. The lid was placed on the bell jar, and a vacuum drawn through. The vacuum was broken 1-2 mins after bubbles began to appear on the leaves and stems and the liquid began to boil. The pot was removed and placed on its side in a plastic potting tray, covered in clingfilm and transferred to continuous white light (100 μmol m^{-2} s^{-1}) at 20°C. The following day the clingfilm was removed and the pot stood upright. Seed from all treated plants was collected as it was ready.

2.11.5 Surface-sterilisation of seed

A small amount of seed was placed onto Whatman 90 mm circular filter paper. The filter paper was folded into quarters, and secured with a paperclip. The filter paper was then washed in 70% EtOH for 2 mins in a Magenta jar, followed by 10% sodium hypochlorite (1.4% (w/v) available chlorine), 0.02% (v/v) Triton X-100 for 10 mins. Finally the filter paper packet was washed 3-4 times in sterile dH_2O in a fume hood. The packet was dried in a fume hood overnight and seed stored in a Petri dish sealed with Micropore® tape.

2.11.6 Selection of kanamycin-resistant transformants

Pooled seed collected from vacuum infiltrated *Arabidopsis* plants, described in 2.11.4, was surface-sterilised as described in 2.11.5, and plated out on 1/2 MS agar containing kanamycin, in Petri dishes. Plates were wrapped with Micropore® tape and transferred to 4°C for 2 days prior to transfer to continuous white light (100 μmol m^{-2} s^{-1}) at 20°C. Seedlings were examined after expansion of the cotyledons for kanamycin resistance. Seedlings which did not bleach were judged to be kanamycin resistant and were transferred to moist compost in continuous white light (100 μmol m^{-2} s^{-1}) at 20°C. Seed was collected from each different T_1 transformant separately, surface sterilised and plated out on 1/2 MS agar containing

kanamycin as before. The segregation ratio (kanamycin resistant:kanamycin sensitive) of seed from each T_1 was determined. T_2 kanamycin-resistant seedlings from T_1 progeny which displayed a 3:1 ratio were transferred to moist compost, and seed from individual T_2 collected and surface-sterilised as before. T_2 progeny was examined for 100% kanamycin resistance, and T_3 seedlings from homozygous lines were used in aequorin experiments.

2.12 Analysis of transgenic aequorin plants

These experiments were undertaken at the University of Edinburgh in collaboration with Dr. G. Baum and Prof. A. Trewavas.

2.12.1 Growth and treatment of transgenic seedlings

Seedlings were germinated on 1/2 MS medium with 0.8% agar in luminometer cuvettes at 21°C in a controlled environment room with a 16 hour white light (25 $\mu mol\ m^{-2}\ s^{-1}$) photoperiod.

Aequorin was reconstituted in vivo by submerging the seedlings in water for a few seconds in order to wet the plants and ensure even distribution of coelenterazine over the seedling. The water was drained and a droplet of 10 μM cp-coelenterazine was placed between the cotyledons. Aequorin was allowed to reconstitute over night in the dark. When using inhibitors, seedlings were submerged in the inhibitor and incubated for 3 hr. Following this treatment the liquid was drained and a droplet of 10 μM cp-coelenterazine with the relevant inhibitor was placed between the cotyledons and left over night in the dark for aequorin to reconstitute.

2.12.2 Light Stimulation

The luminometer cuvette with the seedlings was placed in the cuvette holder of the luminometer in total darkness avoiding any perturbations which might cause mechanical stimulation. The basal luminescence level was measured for several minutes. Then the cuvette holder was turned away from the photomultiplier tube and the seedlings illuminated for the time indicated without taking the tube out of the holder. This way the mechanical stimulation of the plants was minimal and the time required to turn the holder back to the photomultiplier tube and commence measurement was kept to a minimum (about 1 sec). The light source used was a cold white light source (KL750) with a UV-A/blue light filter (BG37) from Schott, Wiesbaden, Germany). The fluence rate was 650 μmol m^{-2} s^{-1}.

In order to calculate [Ca^{2+}] from the luminescence, all of the aequorin remaining after the light treatment was discharged by a series of cold + touch shocks given by fast injection of ice cold water on top of the plants. Because of the high affinity of cp-coelenterazine to calcium more than 90% of the aequorin is discharged by the first combined shock.

2.12.3 Luminescence measurements

Luminescence was measured using a digital chemiluminometer consisting of an EMI photomultiplier tubes model 9829A, with an EMI FACT50 cooling system (Badminton *et al.* 1995).

2.12.4 Calculation of calcium concentration

The following information was kindly provided by Dr. G. Baum.

To determine in vivo calcium concentrations the following equation was adapted from Allen *et al.*, 1997:

$$L/Lmax = ((1 + KR \times [Ca^{2+}]) / (1 + KTR + KR \times [Ca^{2+}]))^3$$

where L is the amount of light (luminescence) per second, Lmax is total amount of light present in the entire sample over the course of the experiment, $[Ca^{2+}]$ as the calculated calcium concentration, KR is the dissociation constant for the first calcium ion to bind to aequorin and KTR is the binding constant of the second calcium ion to bind to aequorin.

It was determined that KR = 26×10^6 M^{-1} and KTR = 57 M^{-1} for cp-coelenterazine by fitting the curve of this equation with the relationship between luminescence and calcium concentration for aequorin reconstituted with cp-coelenterazine (Shimomura *et al.*, 1993). The resulting equation is:

$$[Ca^{2+}](nM) = 58X^{1/3}-1) / 0.026(1-X^{1/3})$$

where X is the amount of light per second divided by the total light emitted after that point until all the aequorin was discharged.

CHAPTER 3: UV-A/BLUE LIGHT SIGNAL TRANSDUCTION REGULATING *rbcS*

3.1 Introduction

A biochemical approach is commonly used to investigate signal transduction pathways in plant and animal cells. In order to introduce pharmacological compounds into plant cells, a single cell approach is preferable, as it ensures easier entry of chemical agonists and antagonists. The *rbcS* genes of *Phaseolus vulgaris* are regulated by a blue light photoreceptor in light-grown primary leaves (Sawbridge *et al.*, 1994). The aim of the experiments in this chapter was to establish whether protoplasts from dark-adapted *P. vulgaris* primary leaves would be suitable for biochemical analysis of blue light-induced *rbcS* gene expression, and to obtain preliminary information on components of the signal transduction pathway based on information already known about other UV/blue light and phytochrome signal transduction pathways in plant cells.

3.2 UV-A/blue light regulation of expression in *Phaseolus vulgaris* leaf protoplasts

Sawbridge *et al.* (1994) described the blue light photoreceptor-mediated expression of *rbcS* genes in light-grown *P.vulgaris* primary leaves. The blue light source used by Sawbridge *et al.* (1994) and in this present study also contains UV-A light (Figure 2.2B). Therefore, this light treatment is referred to as UV-A/blue light in this study. A protoplast isolation method for dark-adapted primary leaves of *P.vulgaris* was developed by Dr N.A.R. Urwin (Glasgow University, UK). The

protoplasts were intact and viable and supported good rates of expression of transfected promoter-reported fusions (N.A.R. Urwin and G.I. Jenkins, in press).

Light-grown *P. vulgaris* plants were dark-adapted for 2 days. Protoplasts were isolated under a green safety light from primary leaves (Section 2.3.2), and are referred to throughout this study as dark-adapted protoplasts. Protoplast viability was determined regularly, and protoplasts with a viability between 70-90% were used. Table 3.1 shows sample measurements from 5 separate experiments. The first objective was to determine whether *rbcS* photoregulation in dark-adapted protoplasts was similar to that observed in the intact leaf. Dark-adapted protoplasts were transferred to different light qualities for 20 hours, and a control sample harvested at the beginning of the experiment. As shown in Figure 3.1, *rbcS* transcripts were not detectable in dark-adapted protoplasts, but show a large induction by white and UV-A/blue light. Transfer of protoplasts to red light did not induce *rbcS* transcript accumulation. This regulation is the same as that observed in the intact primary leaf (Sawbridge *et al.* 1994).

Figure 3.2 shows the induction kinetics of *rbcS* expression in dark-adapted protoplasts transferred to UV-A/blue light. *rbcS* transcript accumulation is transient in a similar manner to intact leaf tissue (Sawbridge, 1993). Transcripts were detectable by 8 hours illumination, peaking at around 12-16 hours, and declining by the 24 hour time point.

3.3 The calcium channel blocker, nifedipine, prevents UV-A/blue light accumulation of *rbcS* transcripts

Calcium is an important second messenger in many plant signal transduction pathways including mechanical stress, low temperature, phytochrome and elicitor signalling (Bush, 1993; Bush, 1995; Johannes *et al.*, 1991; Pooviah and Reddy, 1993). In plant cells, cytosolic calcium concentration is maintained at a constant

Table 3.1 Viablitiy measurements of sample protoplast suspensions

The table shown is the measurement of viablitiy of 10 separate suspensions of protoplasts prepared from dark-adapted *P.vulgaris* primary leaves, using FDA as an indicator. Cells fluorescing green were viable, wheras cells fluorescing red were non-viable.

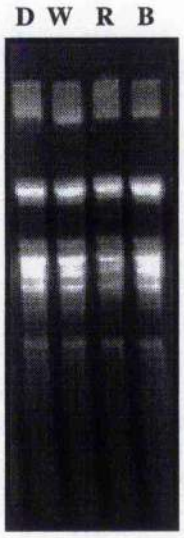
Number of viable protoplasts	Number of non-viable protoplats	% viablity of protoplast suspension
85	29	75
89	11	89
82	7	92
98	25	80
84	26	76

Figure 3.1 UV-A/blue light regulation of *rbcS* in *P.vulgaris* protoplasts

(A) Dark-adapted protoplasts were harvested (D) or transferred to 80 μmol m^{-2} s^{-1} white (W), 80 μmol m^{-2} s^{-1} red (R) or 80 μmol m^{-2} s^{-1} UV-A/blue (B) light. Protoplasts were harvested for RNA extraction after 20 hour exposure. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel,

(B) The gel shown in (A) was blotted to a nylon membrane. The membrane was probed with *P. vulgaris rbcS* cDNA probe, washed and autoradiographed. As a loading control, the membrane was stripped of radioactivity and reprobed with a cDNA encoding the constituitively expressed *P.vulgaris H1* gene product.

(A)



(B)

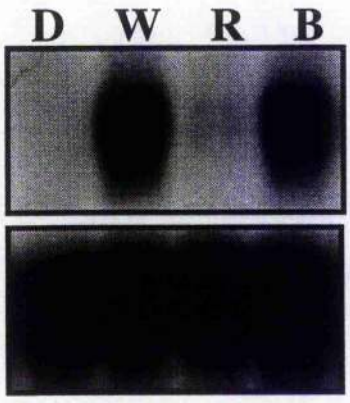
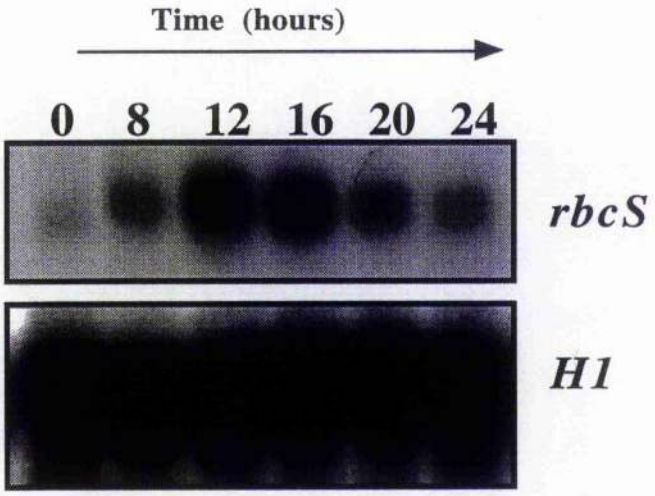


Figure 3.2 Time course of *rbcS* transcript accumulation in UV-A/blue light

Dark-adapted protoplasts were harvested (0) or transferred to 80 μmol m^{-2} s^{-1} UV-A/blue light. Protoplasts were harvested for RNA extraction at the times indicated. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with a *P. vulgaris rbcS'* cDNA probe, washed and autoradiographed. The membrane was then stripped of radioactivity and rehybridised to a *P. vulgaris H1* cDNA probe.


Time (hours)
0
8
12
16
20
24
rbcS
H1

concentration, around 100 nM. This concentration is maintained by calcium efflux transporters and influx channels located at both the plasma membrane and intracellular membranes. Recent research has shown that the cytosolic calcium concentration in plant cells can be dramatically altered by external signals regulating the activity of calcium influx channels (Bush *et al.*, 1995).

Plant calcium channels can be classed by their gating properties. Voltage-gated, stretch-activated and IP_3-gated channels have been identified in plant cells (Bush, 1995). However, perhaps the best studied plant calcium channels are the voltage-gated channels which have been identified at the plasma membrane (Marshall *et al.*, 1994) and the vacuolar membranes (Allen and Sanders, 1994; Johannes *et al.*, 1994). In animal cells, voltage-gated channels are inhibited by the pharmacological agents including the dihydropyridines, for example nifedipine, and phenylalkylamines such as verapamil. In mammalian cells, these calcium channel blockers bind to the $\alpha 1$ subunit of the channel.

Calcium channel blockers have been used to demonstrate the involvement of calcium in plant signal transduction (Monroy and Dhindsa, 1995; Subbaiah *et al.*, 1994; Sedbrook *et al.*, 1996; Haley *et al.*, 1995; Knight *et al.*, 1992; Polisensky and Braam, 1996). In *Arabidopsis* cells, the UV-B and UV-A/blue light induction of *CHS* transcripts is inhibited by nifedipine but not verapamil (Christie and Jenkins, 1996). Recently Frohnmeyer *et al.* (1997) reported that UV-B induced *CHS* and *PAL* expression in parsley cells was also inhibited by nifedipine. In contrast, nifedipine does not inhibit phytochrome induction of *CHS* in soybean cells, but increases *CHS* expression and inhibits phytochrome induction of *CAB* and *fnr* (Bowler *et al.*, 1994b).

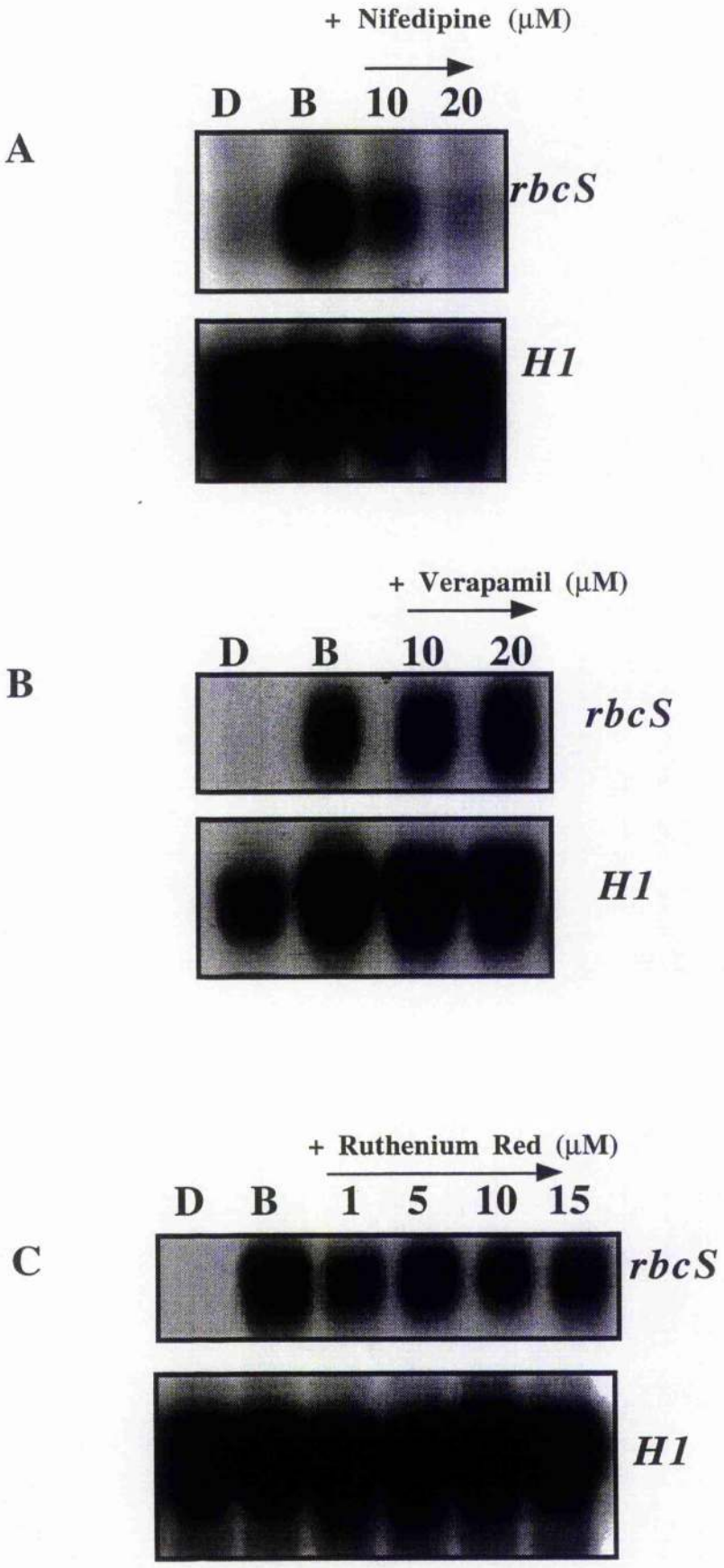
Therefore, the effect of nifedipine and verapamil on the UV-A/blue light induction of *rbcS* in dark-adapted protoplasts was determined. Figure 3.3A shows that incubation of protoplasts with increasing concentrations of nifedipine inhibited *rbcS* transcript accumulation in response to UV-A/blue light. In contrast,

Figure 3.3 Effect of calcium channel blockers on *rbcS* transcript accumulation in response to UV-A/blue light

(A) Dark-adapted protoplasts were harvested (D) or incubated for 30 mins either without (B) or with nifedipine at the concentration indicated prior to illumination with 80 μmol m^{-2} s^{-1} UV-A/blue light. Protoplasts were harvested for RNA extraction after 20 hour exposure. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with a *P. vulgaris rbcS* cDNA probe, washed and autoradiographed. The membrane was then stripped of radioactivity and rehybridised to a *P. vulgaris H1* cDNA probe.

(B) Dark-adapted protoplasts were harvested (D) or incubated for 30 mins either without (B) or with verapamil at the concentration indicated prior to illumination with 80 μmol m^{-2} s^{-1} UV-A/blue light. Protoplasts were harvested for RNA extraction after 20 hour exposure. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. Transcripts were analyzed as in A.

(C) Dark-adapted protoplasts were harvested (D) or incubated for 30 mins either without (B) or with ruthenium red at the concentration indicated prior to illumination with 80 μmol m^{-2} s^{-1} UV-A/blue light. Protoplasts were harvested for RNA extraction after 20 hour exposure. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. Transcripts were analyzed as in A.


+ Nifedipine (μM)
D B 10 20
A
rbcS
H1
+ Verapamil (μM)
D B 10 20
B
rbcS
H1
+ Ruthenium Red (μM)
D B 1 5 10 15
C
rbcS
H1

incubation with verapamil had no inhibitory effect on *rbcS* transcript accumulation (Fig 3.3B).

The UV-A/blue and UV-B light induction of *CHS* in *Arabidopsis* cells is also inhibited by ruthenium red (Christie and Jenkins, 1996) which inhibits plasma membrane and intracellular calcium channels (Marshall *et al.* 1994). Therefore the effect of ruthenium red on UV-A/blue induction of *rbcS* transcripts was determined. As shown in Figure 3.3C, ruthenium red had no effect on UV-A/blue light induced *rbcS* expression.

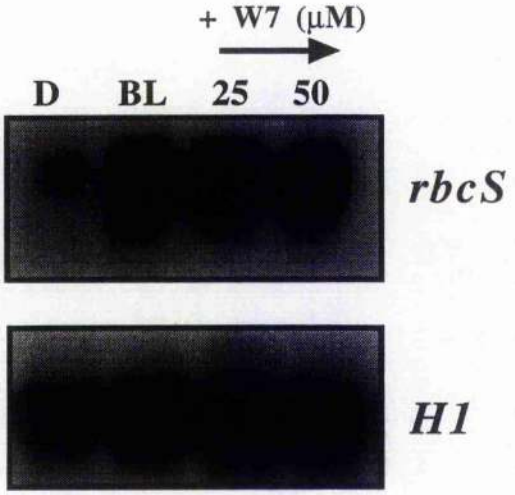
3.4 Calmodulin does not appear to be involved in the UV-A/blue light signalling pathway

As it appears that calcium influx via a nifedipine-sensitive calcium channel is required for *rbcS* expression, the involvement of calmodulin (CaM) was investigated. Calmodulin is an irreversible calcium-binding protein, which when activated by calcium, regulates the activity of numerous plant enzymes including calcium ATPases and Ca^{2+} /CaM-dependent kinases (Roberts and Harmon, 1992). Naphthalene sulphonamide (W-7) is a potent calmodulin inhibitor, and has been used to implicate calmodulin in plant phototransduction pathways (Lam *et al.*, 1989; Frohnmeyer *et al.*, 1997). In *Arabidopsis* cells W-7 was shown to have no effect on *CHS* induction in UV-A/blue light. However, UV-B induced transcript accumulation was inhibited by W-7 in *Arabidopsis* cells and parsley protoplasts (Christie and Jenkins, 1996; Frohnmeyer *et al.*, 1997).

To determine whether calmodulin was involved in UV-A/blue light induced *rbcS* transcript accumulation, the effect of W-7 was determined. As shown in Figure 3.4, W-7 had no effect on UV-A/blue light induced *rbcS* transcript accumulation at up to 50 μM concentration. This result suggests that CaM is not involved in this pathway.

Figure 3.4 Effect of the calmodulin inhibitor, W-7, on *rbcS* transcript accumulation in response to UV-A/blue light

Dark-adapted protoplasts were harvested (D) or incubated for 30 mins either without (BL) or with W-7 at the concentration indicated prior to illumination with 80 μmol m^{-2} s^{-1} UV-A/blue light. Protoplasts were harvested for RNA extraction after 20 hour exposure. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with a *P. vulgaris rbcS* cDNA probe, washed and autoradiographed. The membrane was then stripped of radioactivity and rehybridised to a *P. vulgaris H1* cDNA probe.


+ W7 (μM)
D
BL
25
50
rbcS
H1

3.5 The protein kinase inhibitor, staurosporine, prevents UV-A/blue light induction of *rbcS*

Protein phosphorylation has been implicated in several plant signal transduction pathways, including the UV-A/blue and UV-B induction of *CHS* in *Arabidopsis* cells (Christie and Jenkins, 1996), UV-B induced *CHS* and *PAL* in parsley cells (Frohnmeyer *et al.*, 1997), phytochrome induction of *CAB*, *fnr* and *CHS* in soybean cells (Bowler *et al.*, 1994b) and blue light phototropism in *Arabidopsis* plants (Short and Briggs, 1994; Liscum and Briggs, 1995).

Protein kinases have been extensively studied in plants and most members of mammalian kinase classes have been identified (Stone and Walker, 1995). Commonly used inhibitors in pharmacological studies are staurosporine, a general serine/threonine protein kinase inhibitor, and genistein, a tyrosine protein kinase inhibitor.

Staurosporine has been shown to inhibit UV-A/blue and UV-B induced *CHS* expression in *Arabidopsis* (Christie and Jenkins, 1996) and UV-B induced *CHS* and *PAL* expression in parsley (Frohnmeyer *et al.*, 1997), whereas genistein was shown to have no effect in both systems. Therefore, the effect of these inhibitors on the UV-A/blue light induction of *rbcS* in dark-adapted protoplasts was examined. In Figure 3.5A it can be seen that staurosporine inhibited *rbcS* transcript accumulation. In contrast, genistein had no effect (Figure 3.5B).

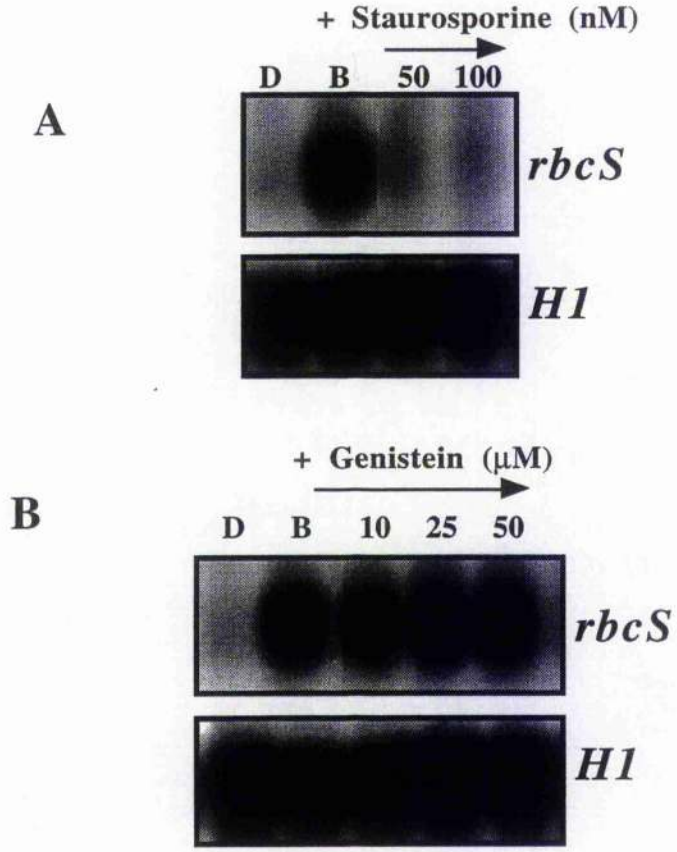
3.6 The protein phosphatase inhibitor, cantharidin, prevents UV-A/blue light induction of *rbcS*

Protein dephosphorylation as well as protein kinase activity is required for the UV-A/blue and UV-B light induction of *CHS* expression in *Arabidopsis* cells (Christie and Jenkins, 1996) and also in light regulation of *rbcS* in maize protoplasts

Figure 3.5 Effect of protein kinase inhibitors on *rbcS* transcript accumulation in response to UV-A/blue light

(A) Dark-adapted protoplasts were harvested (D) or incubated without (B) or with staurosporine at the concentration indicated for 30 mins prior to illumination with 80 μmol m^{-2} s^{-1} UV-A/blue light. Protoplasts were harvested for RNA extraction after 20 hour exposure to light. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with a *P. vulgaris rbcS* cDNA probe, washed and autoradiographed. The membrane was then stripped of radioactivity and rehybridised to a *P. vulgaris H1* cDNA probe.

(B) Dark-adapted protoplasts were harvested (D) or incubated without (B) or with genistein at the concentration indicated for 30 mins prior to illumination 80 μmol $m^{-2}s^{-1}$ UV-A/blue light. Protoplasts were harvested for RNA extraction after 20 hour exposure. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. Transcripts were analyzed as in A.


A
+ Staurosporine (nM)
D
B
50
100
rbcS
H1
B
+ Genistein (μM)
D
B
10
25
50
rbcS
H1

(Sheen, 1993). The effect of the commonly used protein phosphatase 2a inhibitor, cantharidin, was examined. As shown in Figure 3.6, cantharidin inhibited UV-A/blue light induced *rbcS* transcript accumulation in protoplasts. This result indicates a requirement for both protein phosphatase and kinase activity, in the UV-A/blue light induced accumulation of *rbcS* transcripts in dark-adapted protoplasts.

3.7 Cycloheximide inhibits the UV-A/blue light induction of *rbcS*

Protein synthesis has been implicated in photoregulated gene expression, including phytochrome-mediated *CAB* expression (Lam *et al.*,1989), UV-B induced *PR-1* expression (Green and Fluhr, 1995) and UV-A/blue and UV-B induced *CHS* expression (Christie and Jenkins, 1996). Therefore the effect of the protein synthesis inhibitor, cycloheximide, on the UV-A/blue light induction of *rbcS* in dark-adapted protoplasts was examined. As shown in Figure 3.7, the addition of increasing concentrations of cycloheximide to the protoplasts prevented *rbcS* transcript accumulation.

3.8 Discussion

In this chapter, protoplasts from dark-adapted *Phaseolus vulgaris* primary leaves were used as a model system for studying signal transduction components involved in the UV-A/blue light induction of *rbcS*. To investigate the UV-A/blue light signalling pathway regulating *rbcS* the effect of specific pharmacological antagonists was examined. Inhibitors employed in this study have previously been used to study phototransduction in plant cells. In addition to providing preliminary information on components, the findings indicate that the pathway is similar to

Figure 3.6 Effect of the protein phosphatase inhibitor, cantharidin, on *rbcS* transcript accumulation in response to UV-A/blue light

Dark-adapted protoplasts were harvested (D) or incubated without (B) or with increasing concentrations (μM) of cantharidin for 30 mins prior to illumination with 80 μmol m^{-2} s^{-1} UV-A/blue light. Protoplasts were harvested for RNA extraction after 20 hour exposure. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with a *P. vulgaris rbcS* cDNA probe, washed and autoradiographed. The membrane was then stripped of radioactivity and rehybridised to a *P. vulgaris H1* cDNA probe.

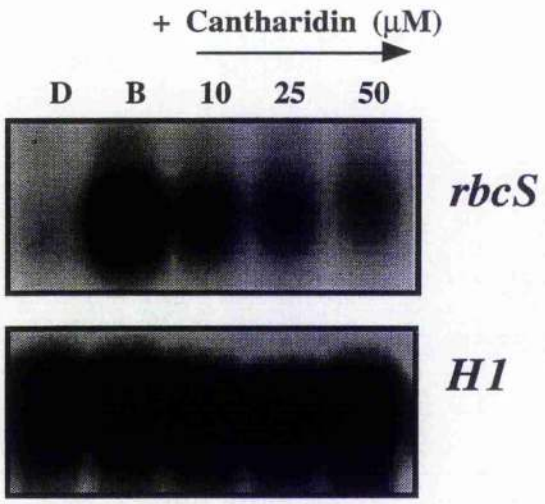
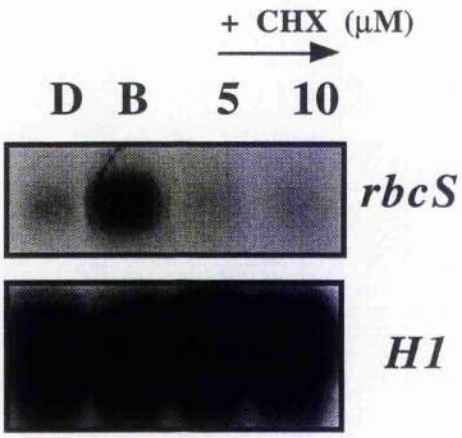

+ Cantharidin (μM)
D
B
10
25
50
rbcS
H1

Figure 3.7 Effect of protein synthesis inhibition on *rbcS* transcript accumulation in response to UV-A/blue light

Dark-adapted protoplasts were harvested (D) or incubated for 30 mins either without (B) or with cycloheximide at the concentration indicated prior to illumination with 80 μmol m^{-2} s^{-1} UV-A/blue light. Protoplasts were harvested for RNA extraction after 20 hour exposure. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with a *P. vulgaris rbcS* cDNA probe, washed and autoradiographed. The membrane was then stripped of radioactivity and rehybridised to a *P. vulgaris H1* cDNA probe.


+ CHX (μM)
D B 5 10
rbcS
H1

other UV/blue light signal transduction pathways coupled to gene expression in other species.

3.8.1 Protoplasts from *P.vulgaris* provide a model system to study UV-A/blue light regulation of *rbcS*

In all experiments involving the analysis of transcripts in this and subsequent chapters, a probe encoding the *H1* gene product (Lawton and Lamb, 1987) was used as a loading control. *H1* transcript levels were unaffected by the pharmacological agents employed in this present study. Unless stated otherwise, the experiments descibed in this and subsequent chapters are representative of the results obtained, and were repeated between two to six times in each case.

Cell cultures and protoplasts from higher plants have been successfully used to study photoregulated transduction components using pharmacological agents (Lam *et al.*, 1989; Bowler *et al.* , 1994a, 1994b; Frohnmeyer *et al.*, 1997; Christie and Jenkins, 1996). The protoplasts used here were evidently physiologically active and responsive to light in the regulation of gene expression. Photoregulation of *rbcS* genes of *P. vulgaris* in light grown primary leaves was reported to be regulated by a blue light photoreceptor (Sawbridge *et al.*, 1994). Regulation of *rbcS* expression in protoplasts isolated from dark-adapted primary leaves is similar to that in mature primary leaves, in both kinetics and the effects of different light qualities (Figures 3.1 and 3.2). UV-A/blue light increased *rbcS* transcript accumulation in dark-adapted protoplasts whereas red light had no effect. Therefore the protoplast system is suitable for the pharmacological dissection of the UV-A/blue light signalling pathway. Table 3.2 summarises the main results from this present chapter.

Table 3.2 Chapter 3 summary

The table shown opposite is a summary of the experimental treatments described in this present chapter and their effect on UV-A/blue light (80 μmol m^{-2} s^{-1}) induced *rbcS* expression in *P.vulgaris* protoplasts.

Treatment	**Effect on UV-A/blue light induced *rbcs* expression**
20 µM nifedipine	Inhibition
20 µM verapamil	No effect
15 µM ruthenium red	No effect
50 µM W-7	No effect
5 nM staurosporine	Inhibition
50 µM genistein	No effect
10 µM cantharidin	Inhibition
5 µM cycloheximide	Inhibition

3.8.2 Preliminary biochemical information on the UV-A/blue phototransduction pathway regulating *rbcS*

Calcium has been implicated as a second messenger in plant phototransduction pathways, including those regulating gene expression. The voltage-gated calcium channel blocker, nifedipine, prevents UV-A/blue light induced *rbcS* transcript accumulation in *P.vulgaris* protoplasts. There was no indication that nifedipine was toxic to the cells and similar concentrations have been found to have no effect on several responses in other systems (Christie and Jenkins, 1996). This result therefore suggests that UV-A/blue light induces a calcium influx into the cytoplasm via voltage-gated calcium channels. However, the cell permeable voltage-gated calcium channel blocker, verapamil, did not inhibit the UV-A/blue light induction of *rbcS*, suggesting that plant cells contain voltage-gated calcium channels which are insensitive to verapamil but sensitive to nifedipine. Similar calcium channel sensitivity was reported by Christie and Jenkins (1996) for the UV-A/blue and UV-B light regulation of *CHS* expression in *Arabidopsis*. However, the location of a UV-A/blue light activated calcium channel is not at present known. Although the calcium channel blocker ruthenium red has been suggested to selectively inhibit intracellular calcium channels, plasma membrane calcium channels which are sensitive to ruthenium red in plant cells have been reported (Marshall *et al.*, 1994). Ruthenium red did not have any significant effect on UV-A/blue light induced *rbcS* transcript accumulation in *P.vulgaris* protoplasts. This is different to the UV-A/blue light induction of *CHS* expression in *Arabidopsis,* which is sensitive to ruthenium red. Additionally, the impermeable calcium channel blocker, lanthanum, did not inhibit *rbcS* expression at concentrations which were not toxic to protoplasts (data not shown). Clearly, further studies are required to identify the cellular location of the nifedipine-sensitive calcium channel.

Calcium regulates the activity of calcium-binding proteins in plant cells (Bush, 1995). However, the calmodulin inhibitor, W-7 did not inhibit the UV-

A/blue pathway. Unfortunately, at the time there was no suitable positive control for these experiments. Although the lack of effect of W-7 could suggest that the compound was not entering the protoplasts, up to 50 μM W-7 was used, which is sufficient to inhibit UV-B light regulated *CHS* expression in *Arabidopsis* cells. Higher concentrations were found to be toxic to the protoplasts, suggesting that the compound was entering the cells. Calmodulin has been shown to be involved in phytochrome and UV-B signal transduction pathways (Lam *et al.*, 1989; Christie and Jenkins, 1996; Frohnmeyer *et al.*, 1997). However, the UV-A/blue light induction of *CHS* in *Arabidopsis* was also insensitive to W-7. This suggests that CaM is not involved in the UV-A/blue light induction of gene expression in plant cells.

As with other phototransduction pathways, protein kinase and protein phosphatase activity appears to be required in the UV-A/blue phototransduction pathway regulating *rbcS* . Staurosporine, a specific inhibitor of serine/threonine kinases inhibited the UV-A/blue light induction of *rbcS* and there was no apparent effect in viability. Since staurosporine inhibits many different classes of plant kinases, it is possible only to speculate where in the signalling pathway kinases are involved. For example, the UV-A/blue pathway is independent of CaM, suggesting that a calcium-activated kinase may act downstream of calcium influx. However, in *Arabidopsis* both the UV-A/blue and UV-B pathways regulating *CHS* gene expression are sensitive to staurosporine, suggesting they may share this part of the pathway, wheras they differ in calmodulin involvement. Therefore, kinase activity could be an early event, possibly upstream of calcium influx and calmodulin involvement. Blue light regulates phosphorylation of a plasma membrane protein as an early step in phototropism in higher plants (Short and Briggs, 1994; Liscum and Briggs, 1995). However, it is possible that more than one UV-A/blue light activated kinase is involved in the pathway.

The tyrosine kinase inhibitor genistein has no effect on the UV-A/blue pathway. Although a control was not available for this experiment, genistein was

found to be toxic to protoplasts at concentrations higher than those shown in Figure 3.5. Additionally, genistein does not inhibit *CHS* expression in *Arabidopsis* cells. This result, together with the nifedipine sensitivity and W-7 insensitivity, suggest that the UV-A/blue light signalling pathways regulating *rbcS* in *P.vulgaris* and *CHS* in *Arabidopsis* are similar.

The inhibition of the UV-A/blue pathway by the protein phosphatase inhibitor, cantharidin suggests that protein dephosphorylation is required. Protein phosphorylation has been implicated in plant phototransduction pathways, including light regulation of *rbcS* in maize protoplasts (Sheen, 1993). This result suggests that the signalling pathway may indeed involve more than one phosphorylation event, with dephosphorylation of a protein required, possibly to inactivate a repressor protein. These inhibitor studies indicate the need for further biochemical analysis of UV-A/blue light regulated protein kinase activity.

The protein synthesis inhibitor, cycloheximide, prevents the UV-A/blue light regulation of *rbcS*. Unfortunaetly no control was available to demonstrate any specificity of this effect, and it is possible that cycloheximide had a general effect on gene expression in the cells. However, cycloheximide had no effect on *TOUCH 3* gene expression in *Arabidopsis* (Christie and Jenkins, 1996). Thus the effect of cycloheximide in the *P. vulgaris* protoplasts may have been specific and could support observations in *Arabidopsis* (Christie and Jenkins, 1996) that cytoplasmic protein synthesis is required for UV-A/blue light signalling. For example, cycloheximide may inhibit synthesis of transcription factors required for *rbcS* promoter activity; the transcription factor CPRF1, which regulates parsley *CHS* promoter activity, is transcriptionally light-regulated in parsley cells and plants (Weisshaar *et al*, 1991; Feldbrugge *et al*, 1994).

It would be interesting to identify and characterise genes which are rapidly induced by UV-A/blue light in *P.vulgaris*. Differential display is a potentially useful technique for identifying genes whose transcription is altered by UV-A/blue light. Preliminary differential display experiments were undertaken in

collaboration with Professor M. Furuya (Hitachi Advanced Research Laboratories, Japan) to identify genes whose transcript accumulation is rapidly altered by UV/blue light. However, due to time constraints we were unable to complete this study for this thesis.

3.8.3 The UV-A/blue light phototransduction pathway appears to be similar to the UV-A/blue light pathway regulating *CHS* in *Arabidopsis* cells

The results of this study suggest that the UV-A/blue light signalling pathway regulating *rbcS* in *P. vulgaris* is similar to the corresponding pathway regulating *CHS* in *Arabidopsis* (Christie and Jenkins, 1996). Both pathways require calcium influx, protein phosphorylation and protein synthesis. Additionally, CaM does not appear to be involved in either pathway.

It is particularly striking that both pathways are affected in a similar manner by the pharmacological inhibitors used. The only difference observed was the insensitivity of UV-A/blue light regulated *rbcS* expression to ruthenium red in this study. Ruthenium red strongly inhibited UV-A/blue light induced *CHS* expression in *Arabidopsis* cells at 50 μM (Christie and Jenkins, 1996). Although there was no appropriate control for experiments in the present study, ruthenium red was found to be toxic to the protoplasts at concentrations greater than 15 μM. This suggests that access to calcium channels was not a problem, but may illustate a significant difference between the two pathways.

Although this study enabled us to gain information on a UV-A/blue light signalling pathway in *P.vulgaris*, there are disadvantages to the experimental approach described in this chapter. Most important is the lack of a suitable control for the inhibitor experiments. Although *H1* transcripts were not affected by the compounds which inhibited *rbcS*, this is not an appropriate control for these

studies. This is because H1 transcripts may be very stable and inhibitory treatments may therefore have little or no effect in the time scale of the experiments. Transcripts induced by some other stimulus would provide a better control. Additionally the time required to isolate viable protoplasts and the incubation time required to detect *rbcS* transcript accumulation was rather long. Incubation of protoplasts in darkness for the equivalent amount of time required for *rbcS* transcript accumulation in the light was found to be toxic to the protoplasts (data not shown). Therefore pharmacological experiments to induce *rbcS* transcript accumulation in darkness would be very difficult.

In contrast, the *Arabidopsis* cell culture (Christie and Jenkins, 1996; Christie, 1996) is an easier model system to study. The experiments are rapid and eliminate the need to isolate protoplasts before each experiment. As well as the preliminary description of components of the UV-A/blue and UV-B light signalling pathways regulating *CHS* expression in *Arabidopsis* (Christie and Jenkins, 1996), the UV-A/blue light photoreceptor, CRY1, has been isolated and characterised (Ahmad and Cashmore, 1993 Lin *et al.*, 1995; Malhotra *et al.*, 1995). Importantly, controls for pharmacological experiments have been described (Christie and Jenkins, 1996; Christie, 1996) and used to demonstrate the specificity of pharmacological inhibitors, including those used in this study. Additionally, the *Arabidopsis hy4* mutant lacking CRY1 is available. Therefore, while the approach described in this Chapter was successful in identifying likely components of a UV-A/blue light signalling pathway, further work on UV/blue light signal transduction pathways in higher plants was carried out using *CHS* regulation in *Arabidopsis* as a model system.

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

4.1 Introduction

Studies in this chapter were carried out using an *Arabidopsis* cell culture system, looking specifically at UV-A/blue and UV-B light induction of the *CHS* and *PAL* genes. Reactions catalysed by *CHS* and *PAL* are described in Figure 1.2. Preliminary information on components of the UV-A/blue and UV-B light signalling pathways regulating *CHS* have been described (Christie and Jenkins, 1996). *CHS* and *PAL* are induced in the *Arabidopsis* cells specifically by UV-A/blue and UV-B light (Christie and Jenkins, 1996; Christie, 1996) similar to the induction in mature leaf tissue (Jackson *et al.*, 1995; Fuglevand *et al.*, 1996). The aim of the experiments in this section was to further characterise the UV-A/blue and UV-B light signalling events in *Arabidopsis* cells using a biochemical approach.

4.2 Kinetics of UV-B and UV-A/blue light induction of *CHS* are different

The *Arabidopsis* cell culture used in these experiments was described previously (May and Leaver, 1993; Christie and Jenkins, 1996). When grown under a continuous, low fluence rate of white light (20 μmol m^{-2} s^{-1}) *CHS* transcripts do not accumulate (Christie and Jenkins, 1996) whereas transfer to continuous illumination with either UV-A/blue or UV-B light induces *CHS* transcript accumulation within approximately 4 hours. No difference in the kinetics was

evident between the light treatments (Christie and Jenkins, 1996). Frohnmeyer *et al.* (1997) recently described the effect of UV-B light treatments on *CHS* expression in parsley cells. They found that a 30 min UV-B treatment followed by transfer to darkness for 6 hours induced a comparable *CHS* and *PAL* transcript accumulation to 6 hours continuous UV-B light. However, 15 mins of UV-B followed by darkness did was not sufficient to induced *CHS* and *PAL* transcripts. In order to investigate whether a similar response is present for UV-A/blue and UV-B light induced *CHS* expression in *Arabidopsis* cells, the effect of brief UV-A/blue and UV-B light treatments was examined. Following the light treatments, cells were returned to low white light and harvested 6 hours after the start of illumination. As shown in Figure 4.1, although *CHS* expression requires at least one hour of UV-A/blue light before transcript accumulation is subsequently detected, UV-B induces *CHS* transcript accumulation within only 5 mins of UV-B exposure. The level of expression continued to increase with increasing UV-B exposure. This result illustrates the difference between the two phototransduction pathways.

4.3 Ferricyanide and DCPIP inhibit the UV-A/blue and UV-B induction of *CHS* and *PAL*

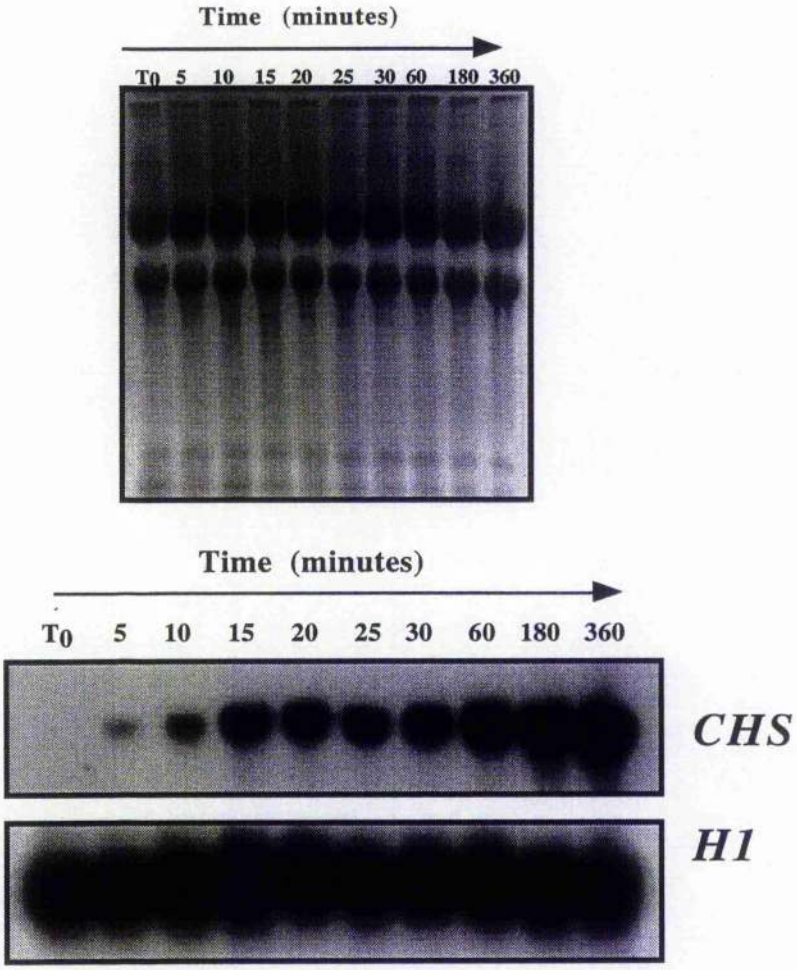
The photoreceptor CRY1 has striking sequence homology with microbial DNA photolyases (Ahmad and Cashmore, 1993), which are flavoprotein enzymes that repair DNA via electron transfer to pyrimidine dimers (Heelis *et al.*, 1993; Sancar, 1994). However, CRY1 has no detectable photolyase activity (Malhotra *et al.*, 1995) suggesting that CRY1 may initiate signal transduction pathways via electron transfer. It is well documented that blue light induces redox processes at the plasma membrane (Asard *et al.*, 1995; Rubinstein and Luster, 1993). The blue light-inducible b-type cytochrome has been extensively studied, although a

Figure 4.1 Time course of UV-B and UV-A/blue light on *CHS* transcript accumulation.

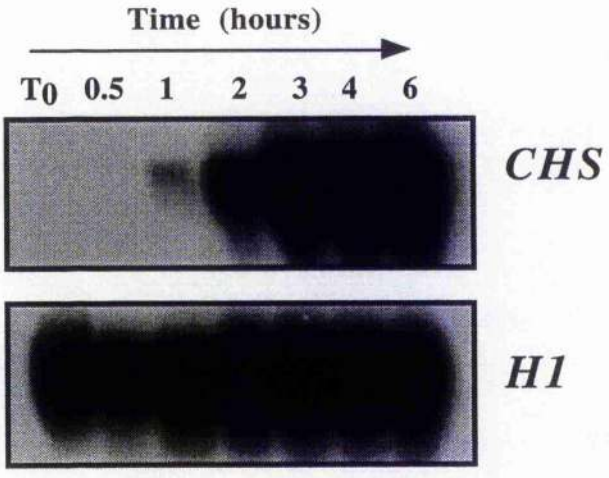
(A) Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (T_0) or transferred to 3 μmol m^{-2} s^{-1} UV-B light for the times indicated and subsequently returned to 20 μmol m^{-2} s^{-1} white light. Cells were harvested for RNA extraction 6 hours after the start of illumination. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel as shown in figure (A). The gel was blotted on 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 in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (T_0) or transferred to 80 μmol m^{-2} s^{-1} UV-A/blue light for the times indicated and subsequently returned to 20 μmol m^{-2} s^{-1} white light. Cells were harvested for RNA extraction 6 hours after the start of illumination and transcripts were analysed as in A.

(A)


Time (minutes)
T0 5 10 15 20 25 30 60 180 360
Time (minutes)
T0 5 10 15 20 25 30 60 180 360
CHS
H1

(B)


Time (hours)
T0 0.5 1 2 3 4 6
CHS
H1

physiological role for this has not been reported (Asard *et al.*, 1994). However, blue light regulated redox processes have been implicated in other responses such as proton pumping in guard cells (Gautier *et al.*, 1992; Vani and Raghavendra, 1992), plasma membrane protein phosphorylation (Hager *et al.*, 1993), cell wall peroxidase (Shinkle and Jones, 1988) and photopolarization in *Fucus* zygotes (Berger and Brownlee, 1994).

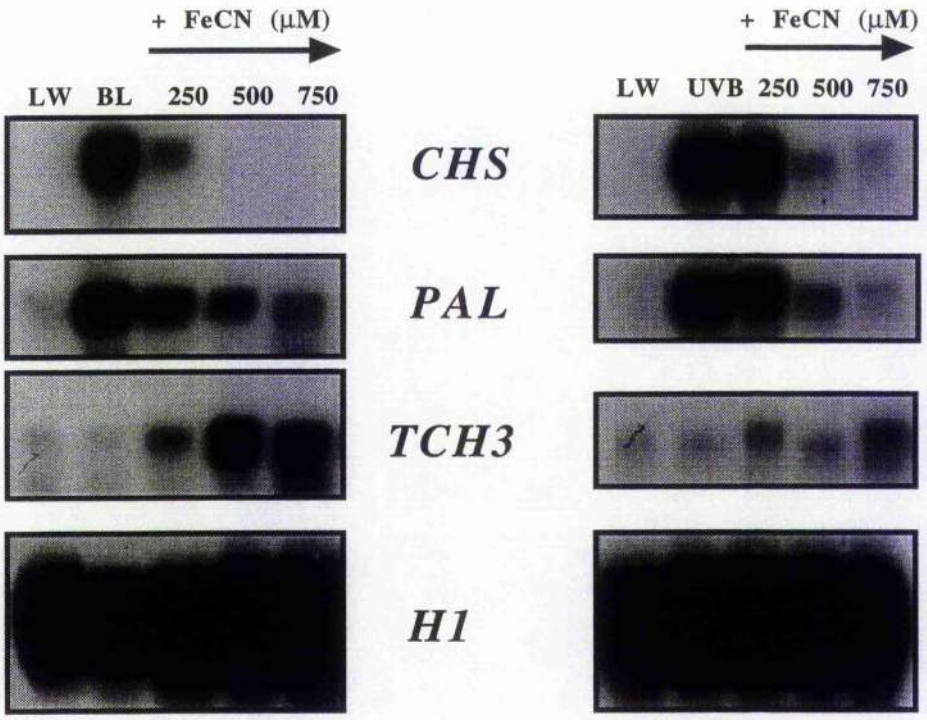
Plant plasma membrane redox processes have been studied using electron acceptors with high redox potentials, such as ferricyanide (Vani and Raghavendra, 1992; Berger and Brownlee, 1994; Dharmawardhane *et al*, 1989; Barr *et al*, 1990). Therefore, the effect of the impermeable electron acceptor, potassium ferricyanide (FeCN) was used to determine if plasma membrane electron transport is required in UV-B and UV-A/blue light induced *CHS* and *PAL* transcript accumulation. Additionally, dichlorophenol indophenol (DCPIP) was also used. Unlike FeCN, DCPIP reportedly is cell permeable. FeCN and DCPIP stock solutions were made up fresh immediately before each experiment, and added to the cells immediately prior to transfer to UV-A/blue or UV-B light at the concentrations shown in Fig 4.2. Both FeCN and DCPIP inhibited the transcript accumulation of *CHS* and *PAL* in both UV-A/blue and UV-B light. The inhibition of both the UV-A/blue and UV-B light induction of *CHS* and *PAL* with the cell impermeable acceptor, FeCN, suggests that plasma membrane redox processes are required.

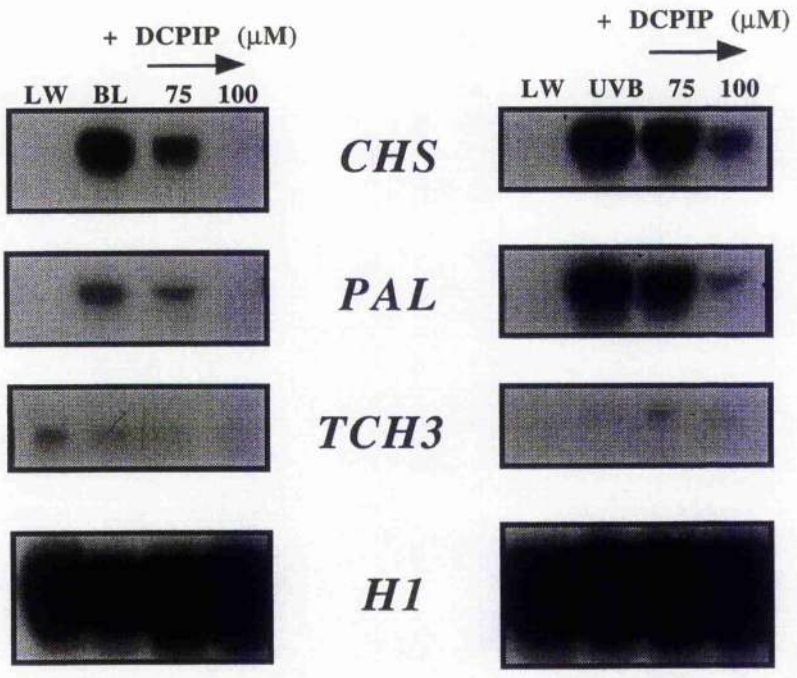
Interestingly, it was also observed that FeCN, but not DCPIP, induced expression of *TOUCH 3 (TCH3)*, the calmodulin-like protein gene (Braam and Davis, 1990; Braam, 1992; Sistrunk *et al.*, 1994). *TCH3* is induced by many different environmental stimuli, such as mechanical stress and darkness, all of which are thought to mediate their responses via an increase in cytosolic calcium. Thus FeCN did not have a general inhibitory effect on gene expression in the cells.

Figure 4.2 Effect of electron acceptors on *CHS*, *PAL*, and *TCH3* transcript accumulation by UV-B and UV-A/blue light

(A) Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) or transferred, without (BL, UVB) or with increasing concentrations (μM) of FeCN, to illumination with 3 μmol m^{-2} s^{-1} UV-B or 80 μmol m^{-2} s^{-1} UV-A/blue light. Cells were harvested for RNA extraction after 6 h exposure. Equal amounts of RNA (20 μg) were separated on a 1.3% denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. The membrane was stripped of radioactivity and reprobed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis TCH3* probe, washed and subjected to autoradiography. Finally, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.

(B) Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) or transferred, without (BL, UVB) or with increasing concentrations (μM) of DCPIP, to illumination with 3 μmol m^{-2} s^{-1} UV-B or 80 μmol m^{-2} s^{-1} UV-A/blue light. Cells were harvested for RNA extraction after 6 h exposure. Transcripts were analysed as in (A).


+ FeCN (μM)
LW BL 250 500 750
+ FeCN (μM)
LW UVB 250 500 750
CHS
PAL
TCH3
H1


+ DCPIP (μM)
LW BL 75 100
+ DCPIP (μM)
LW UVB 75 100
CHS
PAL
TCH3
H1

4.4 Ferricyanide and DCPIP have no effect on induction of *PAL* by cantharidin

It was previously shown that the protein phosphatase inhibitor, cantharidin, induces *PAL* gene expression in *Arabidopsis* cells (Christie and Jenkins, 1996). This response was used as a control to demonstrate that pharmacological agents which inhibited UV/blue light induced *CHS* expression, were not having a general effect on gene expression. To determine that the effect of FeCN and DCPIP is not a general toxic effect, cells were incubated with 200 μM cantharidin in low white light in the presence of 750 μM FeCN or 100 μM DCPIP. As shown in Figures 4.3A and 4.3B, concentrations of FeCN and DCPIP that inhibit the UV-A/blue and UV-B light induction of *CHS* and *PAL*, have no equivalent effect on the cantharidin induction of *PAL* .

4.5 FeCN rapidly induces *TCH3* in low white light

Addition of FeCN to *Arabidopsis* cells stimulated *TCH3* and inhibited *CHS* expression in UV-A/blue and UV-B light. However, as shown in Figure 4.4 *TCH3* expression was induced in cells incubated with 750 μM FeCN transferred to either low white, UV-A/blue or UV-B light. The kinetics of *TCH3* induction by ferricyanide addition to the *Arabidopsis* cells was determined. Figure 4.5 shows that FeCN strongly induced *TCH3* within 2 hours, and the increase was transient, since transcripts decreased by 6 hours. Therefore, as FeCN is inducing *TCH3* transcript accumulation in low white as well as UV-A/blue and UV-B light, this suggests that FeCN induction of *TCH3* is independent of FeCN inhibition of UV-A/blue and UV-B light regulated *CHS* and *PAL* expression.

Figure 4.3 Effect of electron acceptors on the cantharidin induction of *PAL* gene expression.

(A) Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) without (-) or with 750 μM FeCN in the prescence of 200 μM cantharidin. Cells were harvested for RNA extraction after 6 hours. Equal amounts of RNA (20 μg) were separated on a 1.3% denaturing agarose gel and blotted on to a nylon membrane. The membrane 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.

(A) Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) without (-) or with 100 μM DCPIP in the prescence of 200 μM cantharidin. Cells were harvested for RNA extraction after 6 hours. Transcripts were analyzed as in (A).

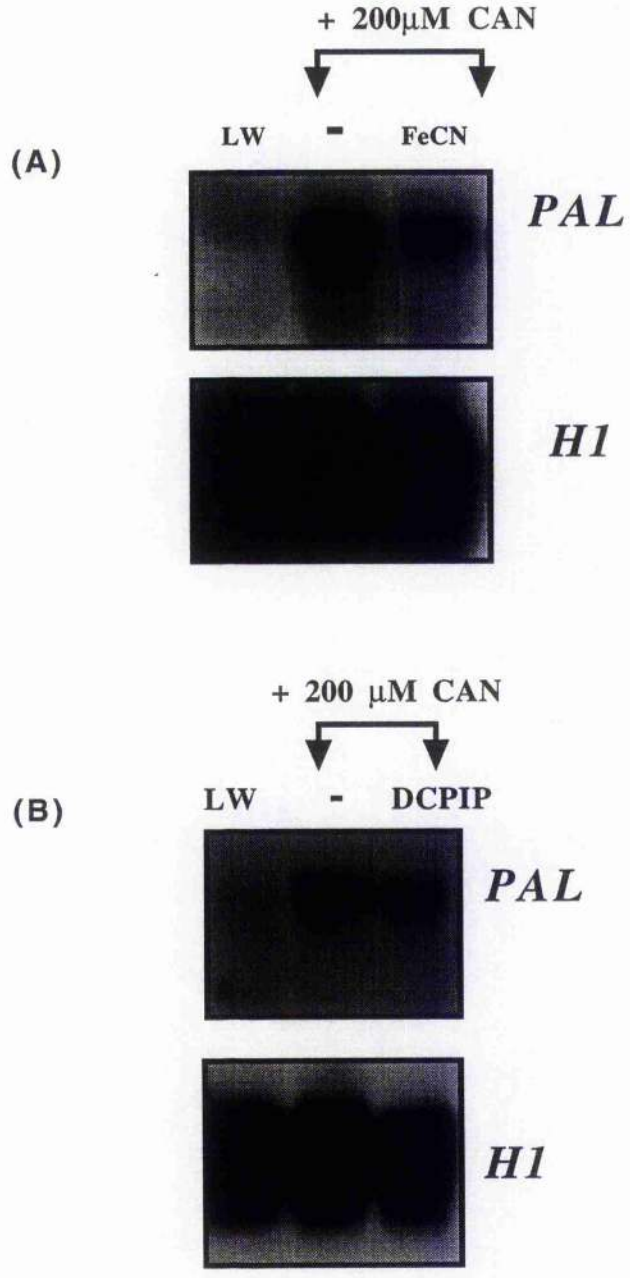

+ 200μM CAN
LW - FeCN
(A)
PAL
H1
+ 200 μM CAN
LW - DCPIP
(B)
PAL
H1

Figure 4.4 Effect of FeCN on *TCH3* gene expression in low white, UV-B and UV-A/blue light

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white, 80 μmol m^{-2} s^{-1} UV-A/blue or 3 μmol m^{-2} s^{-1} UV-B light without (LW, UVB, BL) or with (+FeCN) 750 μM FeCN. Cells were harvested for RNA extraction after 6 hours. Equal amounts of RNA (20 μg) were separated on a 1.3% denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with an *Arabidopsis TCH3* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Finally, the membrane was stripped and reprobed with a*Phaseolus vulgaris H1* probe.

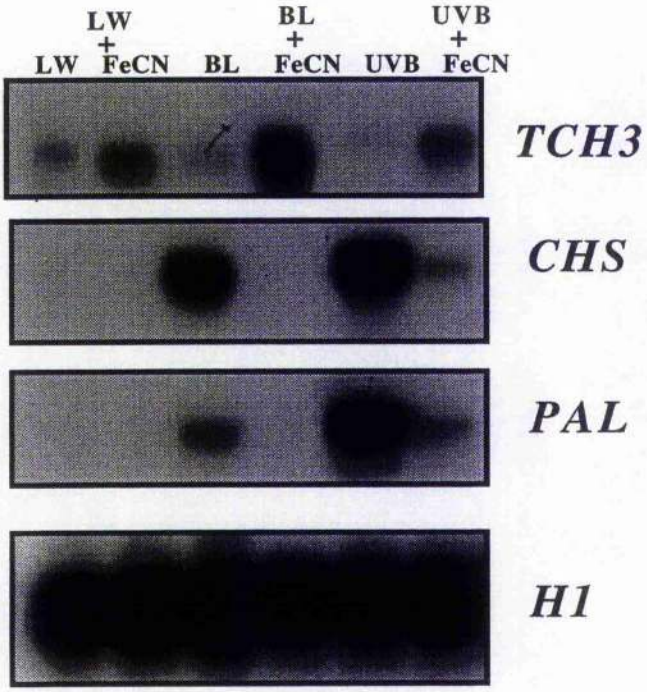
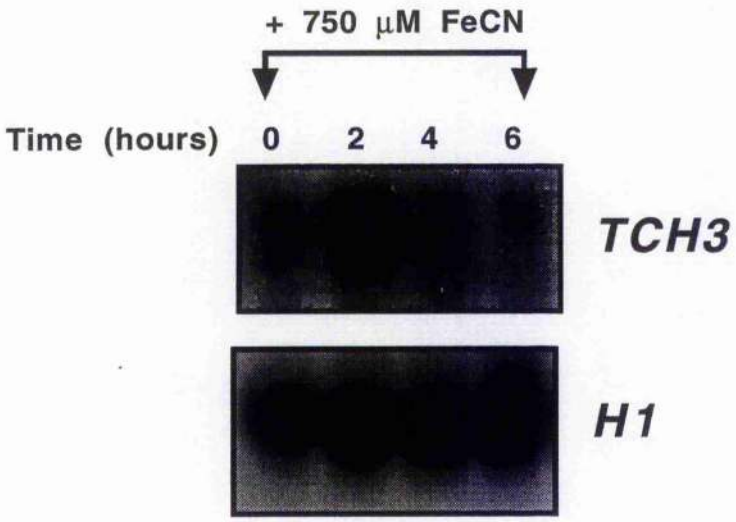

LW
+
LW FeCN
BL
BL
+
FeCN
UVB
UVB
+
FeCN
TCH3
CHS
PAL
H1

Figure 4.5 Time course of FeCN induction of *TCH3* transcripts in low white light

Cells grown in 20 μmol m^{-2} s^{-1} were incubated in 20 μmol m^{-2} s^{-1} white light without (-) or with (+) 750 μM FeCN. Cells were harvested for RNA extraction at the times indicated. Equal amounts of RNA (20 μg) were separated on a 1.3% denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with an *Arabidopsis TCH3* probe, washed and subjected to autoradiography. Finally, the membrane was stripped and reprobed with a *Phaseolus vulgaris H1* probe.


+ 750 μM FeCN
Time (hours) 0 2 4 6
TCH3
H1

4.6 Calcium channel blockers, lanthanum and nifedipine, do not prevent the ferricyanide induction of *TCH3* in UV-B and UV-A/blue light

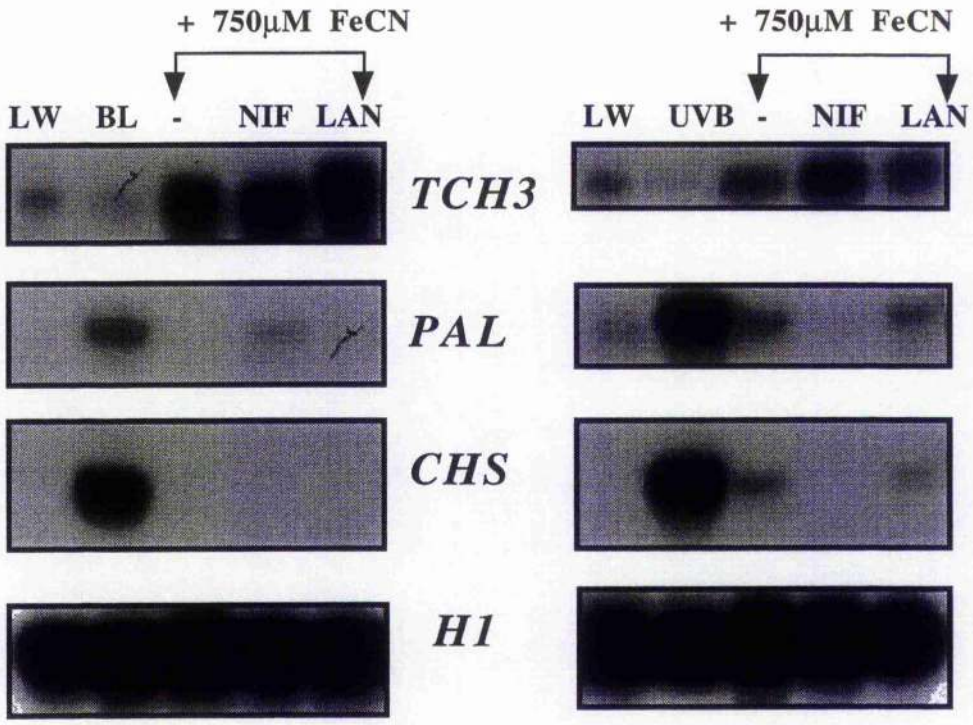
Ferricyanide reduction by intact cells or tissues stimulates acidification of the external medium and plasma membrane depolarization (Marre *et al.*, 1988; Crane and Barr, 1989). This depolarization most likely stimulates ion fluxes across the plasma membrane, possibly activating voltage-gated calcium channels. Influx of calcium via voltage-gated channels could result in *TCH3* induction. Therefore the effect of calcium channel blockers, nifedipine and lanthanum, on ferricyanide induced *TCH3* transcript accumulation was determined. Figure 4.6 demonstrates that neither inhibitor had any effect on ferricyanide induced *TCH3* induction.

4.7 Diphenylene iodonium inhibits both the UV-B and the UV-A/blue light induction of *CHS* and *PAL*

Plasma membrane electron transport appears to be involved in both UV-A/blue and UV-B induced *CHS* and *PAL* expression in *Arabidopsis* cells. Redox activity at the plasma membrane of plant cells has been shown to regulate processes such as ion transport, hormone uptake, iron reduction and uptake (Rubinstein and Luster, 1993). Particularly interesting is the generation of reactive oxygen species (ROS) at the plasma membrane by electron transfer to molecular oxygen. Reduction of molecular oxygen via electron donation results in the formation of superoxide (O^{2-}) and hydrogen peroxide (H_2O_2), and these ROS have been implicated in signalling pathways in plant cells, including the regulation of gene expression (Levine *et al.*, 1994; May and Leaver, 1993). The possibility that UV-A/blue and UV-B light induced redox activity could regulate the production of ROS is particularly intriguing. Several oxidoreductase systems have been identified at the

Figure 4.6 Effect of lanthanum and nifedipine on ferricyanide induced accumulation of *TCH3* transcript

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) without (BL, UVB) or with 50μM nifedipine (NIF) or 10 mM lanthanum (LAN) for one hour prior to addition of 750 μM ferricyanide, and transfer to 3 μmol m^{-2} s^{-1} UV-B or 80 μmol m^{-2} s^{-1} UV-A/blue light. Cells were harvested for RNA extraction after 6 hours. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane 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, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. Finally, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.


+ 750μM FeCN
LW BL - NIF LAN
+ 750μM FeCN
LW UVB - NIF LAN
TCH3
PAL
CHS
H1

plasma membrane of plant cells, including a superoxide generating NADPH oxidase and plasma membrane bound peroxidases (Crane and Barr, 1989; Rubinstein and Luster, 1993; Vianello and Macri, 1991).

The O_2^- generating NADPH oxidase has recently received interest, particularly in its proposed role in pathogen-activated signal transduction. Upon pathogen attack, plant cells rapidly stimulate H_2O_2 production, a response termed the oxidative burst (Lamb and Dixon, 1997). Recently, there has been speculation that a plant NADPH oxidase, similar to the superoxide generating enzyme found in mammalian neutrophil cells, is responsible for this oxidative burst (Lamb and Dixon, 1997). In activated mammalian neutrophils, an electron from cytosolic NADPH is transferred across the plasma membrane to molecular oxygen, to form superoxide outside the cell, and subsequently hydrogen peroxide. Evidence is accumulating to support the similarity of plant NADPH oxidase and mammalian NADPH oxidases (Dwyer *et al.*, 1996); most of this is immunological evidence (Levine *et al.*, 1994; Xing *et al.*, 1997, Kieffer *et al.*, 1997). However, a gene from rice with homology to the mammalian gp91 subunit of NADPH oxidase was recently isolated (Groom *et al.*, 1996). Although NADPH oxidase activity is commonly associated with pathogen-induced signalling, UV-B was recently demonstrated to activate NADPH oxidase activity in *Arabidopsis* (Rao *et al.*, 1996).

In neutrophil cells, the NADPH oxidase is inhibited by the lipophilic suicide substrate diphenylene iodonium (DPI). DPI has been used to study superoxide generating NADPH oxidase in plant cells (Levine *et al.*, 1994) and to implicate ROS in plant signalling pathways. The inhibitory mode of action of DPI was recently described for the neutrophil NADPH oxiase (O'Donnell *et al.* , 1993). It was proposed that DPI preferentially inhibits reduced flavins by interfering with electron transport. Therefore, an experiment was undertaken to determine whether DPI inhibited UV-A/blue or UV-B light induced *CHS* and *PAL* transcript accumulation.

Figure 4.7 shows that DPI inhibited both UV-A/blue and UV-B induced *CHS* and *PAL* transcript accumulation. This result suggests that UV/blue light activates a flavoprotein, possibly even NADPH oxidase activity, as a necessary component of the signalling pathway regulating *CHS* and *PAL* gene expression. Additionally, DPI also induced *TCH3* gene expression in UV/blue light, indicating that DPI did not have a general inhibitory effect on gene expression.

4.8 DPI has no effect on cantharidin induction of *PAL* transcripts in low white light

As a further control to examine whether DPI generally affected gene expression, an experiment was undertaken to observe whether DPI also inhibited the cantharidin induction of *PAL*. Cells were preincubated with DPI for one hour in low white light, after which time 200 μM cantharidin was added to the cells. Figure 4.8 shows that DPI had no effect on the cantharidin induction of *PAL* transcript accumulation. This further demonstrates that DPI is not having a general deleterious effect on gene expression in *Arabidopsis* cells.

4.9 DPI rapidly induces *TCH3* in low white light

The previous experiments with FeCN demonstrated that induction of *TCH3* by inhibition of plasma membrane electron transport was rapid, with a strong induction by 2 hours. Therefore the kinetics of *TCH3* induction by DPI were determined. Figure 4.9 demonstrates that DPI induces *TCH3* with similar kinetics to the FeCN induction of *TCH3*, with transcript induced by 2 hours. Again this induction was transient, and was decreased by 6 hours.

Figure 4.7 Effect of DPI on *CHS* and *PAL* transcript accumulation in UV-B and UV-A/blue light

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) for one hour without (BL, UVB) or with DPI at the concentrations shown prior to transfer to 3 μmol m^{-2} s^{-1} UV-B or 80 μmol m^{-2} s^{-1} UV-A/blue light. Cells were harvested for RNA extraction after 6 hours. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane 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, washed and subjected to autoradiography. The membrane was stripped of radioactivity and reprobed with an *Arabidopsis TCH3* probe, washed and subjected to autoradiography. Finally the membrane was stripped and reprobed with a *Phaseolus vulgaris H1* probe.

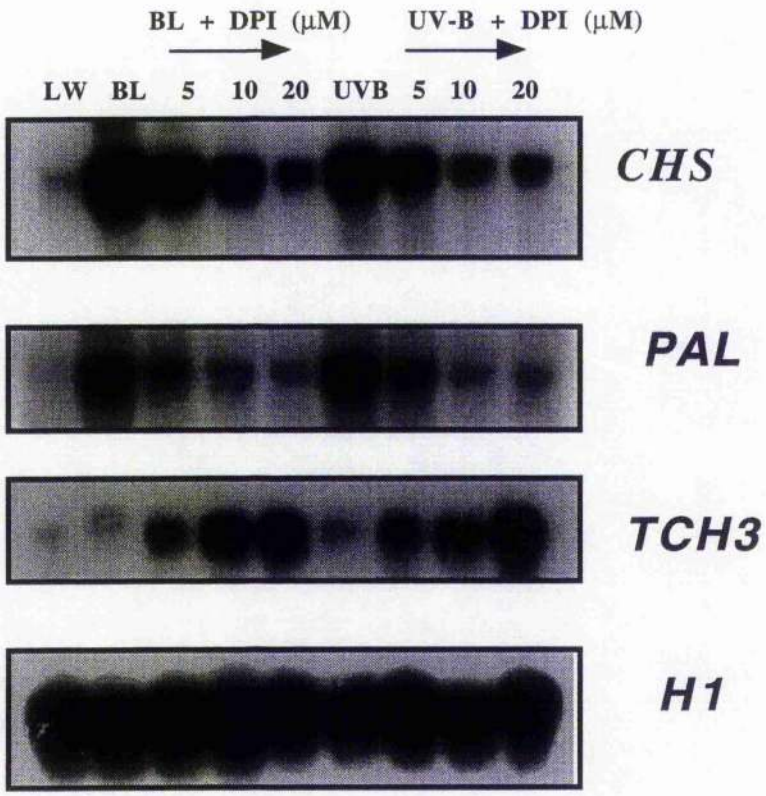

BL + DPI (μM)
UV-B + DPI (μM)
LW BL 5 10 20 UVB 5 10 20
CHS
PAL
TCH3
H1

Figure 4.8 Effect of DPI on the cantharidin induced accumulation of *PAL* transcripts in low white light

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) without (-) or with 20 μM DPI for one hour prior to the addition of 200 μM cantharidin. Cells were harvested for RNA extraction after 6 hours. Equal amounts of RNA (20 μg) were separated on a 1.3% denaturing agarose gel and blotted on to a nylon membrane. The membrane 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.

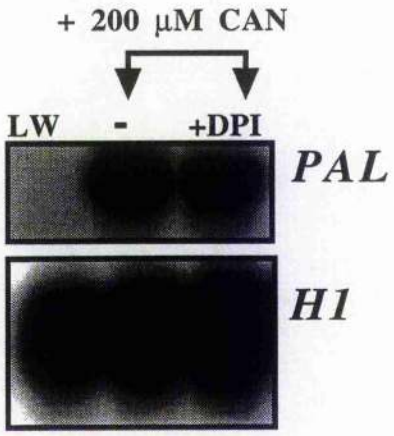
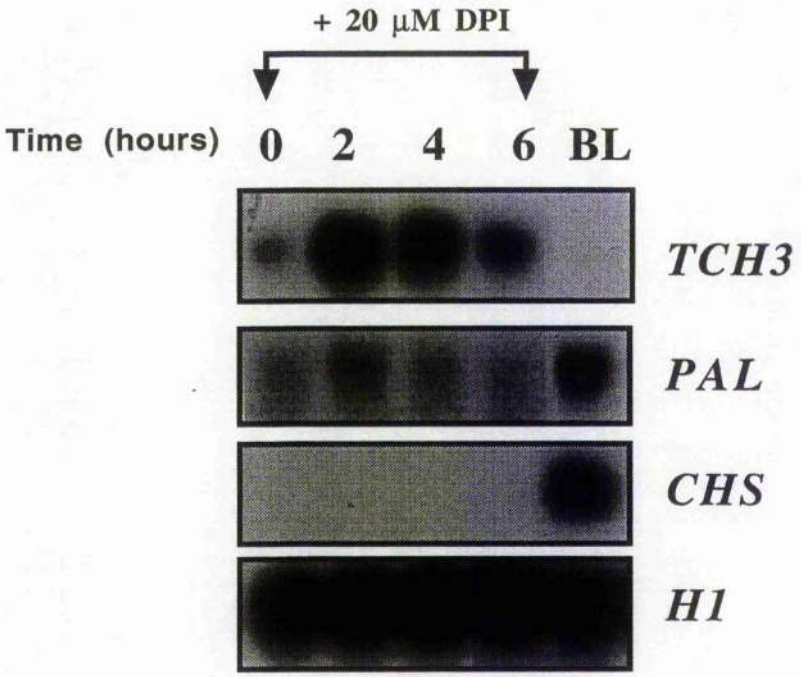

+ 200 μM CAN
LW
-
+DPI
PAL
H1

Figure 4.9 Effect of DPI in low white light on *TCH3* and *PAL* transcript accumulation

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light in the prescence of DPI for the time indicated or transferred to 80 μmol m^{-2} s^{-1} UV-A/blue light for 6 hours (BL). After the appropriate time, cells were harvested for RNA extraction. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with an *Arabidopsis TCH3* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. The membrane was then stripped of radioactivity and reprobed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Finally, the membrane was stipped and reprobed with a *Phaseolus vulgaris H1* probe.


+ 20 μM DPI
Time (hours)
0
2
4
6
BL
TCH3
PAL
CHS
H1

4.10 UV-A/blue and UV-B light prevent calcium induction of *TCH3*

Experiments presented earlier clearly show that FeCN and DPI when added to the cell culture induce *TCH3* expression. Since *TCH3* expression is induced by increased cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$), these compounds are probably altering calcium ion fluxes at the plasma membrane, resulting in an increased $[Ca^{2+}]_{cyt}$ and induction of *TCH3* expression. Furthermore, it was possible that the increased $[Ca^{2+}]_{cyt}$ caused the inhibition of *CHS* and *PAL* expression in UV-A/blue and UV-B light. Therefore the effect of artificially increasing $[Ca^{2+}]_{cyt}$, using a calcium ionophore on the UV-A/blue or UV-B induction of *CHS* was determined.

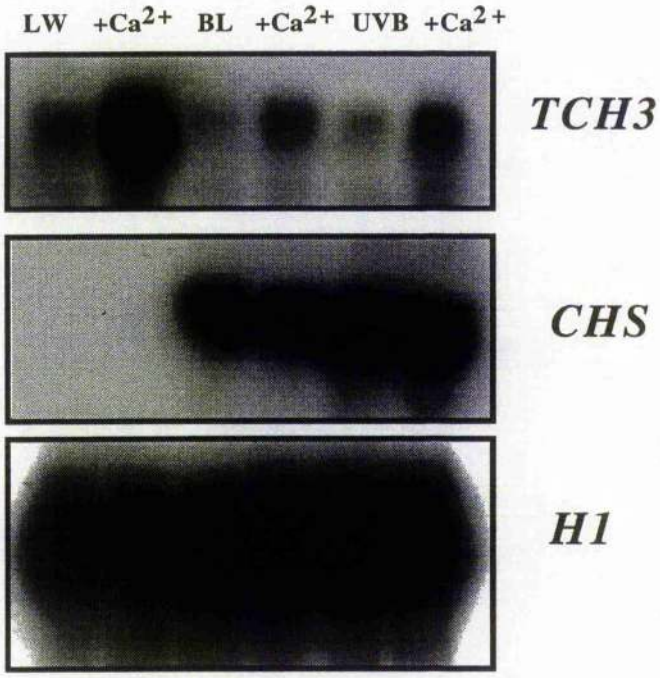
Cells were incubated in low white light with the ionophore, A23187, and calcium for one hour, prior to transfer to UV-A/blue or UV-B light for 6 hours. Figure 4.10 shows that the ionophore/Ca^{2+} treatment induced *TCH3* expression wheras the UV/blue light induction of *CHS* expression was not affected by this pre-treatment with calcium, induction of *TCH3* was significantly reduced in cells transferred to UV-A/blue or UV-B light. This unexpected result suggests that when *Arabidosis* cells with an ionophore-mediated increase in $[Ca^{2+}]_{cyt}$ are transferred to either UV-A/blue or UV-B light, the result is a decreased $[Ca^{2+}]_{cyt}$ and reduced*TCH3* expression.

4.11 The calcium pump inhibitor, erythrosin-B, inhibits the UV-A/blue but not the UV-B induction of *CHS*

The previous experiment implies that UV-A/blue and UV-B light could activate efflux of calcium from the cytoplasm. Plant cells contain Ca^{2+}-ATPases, antiports and uniports to remove calcium from the cytoplasm either across the plasma

Figure 4.10 Lack of effect of increased $[Ca^{2+}]_{cyt}$ on the UV-A/blue and UV-B light induction of *CHS* transcript accumulation

Cells grown in 20 μmol m^{-2} s^{-1} white light were pre-incubated in 20 μmol m^{-2} s^{-1} white light, without (LW, UVB, BL) or with (+Ca^{2+}) 10 μM A23187 and 10 mM $CaCl_2$ for one hour prior to transfer to 80 μmol m^{-2} s^{-1} UV-A/blue or 3 μmol m^{-2} s^{-1} UV-B light. Cells were harvested for RNA extraction after 6 hours. Equal amounts of RNA (20 μg) were separated on a 1.3% denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with an *Arabidopsis TCH3* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Finally, the membrane was stripped and reprobed with a *Phaseolus vulgaris H1* probe.


LW +Ca2+ BL +Ca2+ UVB +Ca2+
TCH3
CHS
H1

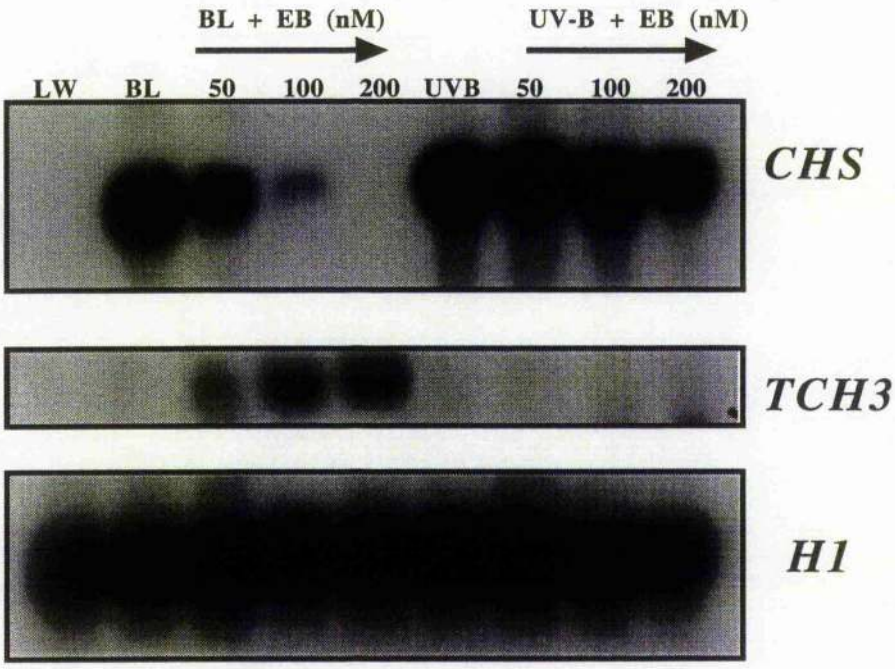
membrane or to intracellular organelles (Bush, 1995). Probably the primary mechanism is the P-type Ca^{2+}-ATPase, which like its counterpart in animal cells can be found at both the plasma membrane and intracellular membranes (Evans *et al.*, 1994). Therefore an experiment was undertaken to determine whether Ca^{2+}-ATPase activity is required for the UV-A/blue and UV-B light induction of *CHS* expression.

Mammalian calcium ATPases can be clearly divided into two categories. The plasma membrane (PM) type is activated by calmodulin and its activity is increased by lanthanum. The ER type, located at the sarcoplasmic and endoplasmic reticulum is not stimulated by calmodulin, and is inhibited by lanthanum. Plant cells clearly differ from mammalian cells in that calmodulin activated Ca^{2+}-ATPases are present in intracellular membranes (Askerlund, 1997; Evans, 1994) and plasma membrane (Rasi-Caldogno *et al.*, 1995). Additionally, pharmacological agents which effectively inhibit mammalian ER-type Ca^{2+}-ATPase have little or no effect on plant Ca^{2+}-ATPases (Thomson *et al.*, 1994). However, one compound which is effective at low concentrations is the fluorescein-derivative, erythrosin B (EB), which will inhibit both plasma membrane and intracellular Ca^{2+}-ATPase in the nanomolar concentration range, but not H^{+}-ATPases, which require micromolar concentrations (Evans *et al.*, 1994; Thomson *et al.*, 1994). At present no inhibitor is available which can discriminate between Ca^{2+}-ATPases in the plant cell plasma membrane and intracellular locations.

Figure 4.11 demonstrates that EB, at 50-100 nM, inhibits *CHS* transcript accumulation in UV-A/blue light. Furthermore, *TCH3* expression was stimulated in UV-A/blue light, indicating that EB was increasing $[Ca^{2+}]_{cyt}$ by blocking Ca^{2+}-ATPase activity. In contrast, EB had no effect on UV-B induced *CHS* transcript accumulation, and *TCH3* was not observed to increase in these cells, suggesting that the proposed UV-B light activated calcium efflux activity is not inhibited by EB.

Figure 4.11 Effect of erythrosin-B on *CHS* and *TCH3* transcript accumulation in UV-B and UV-A/blue light

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) without (BL, UVB) or with EB at the concentrations shown for one hour prior to transfer to 3 μmol m^{-2} s^{-1} UV-B or 80 μmol m^{-2} s^{-1} UV-A/blue light. Cells were harvested for RNA extraction after 6 hours. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted ON to a nylon membrane. The membrane 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, washed and subjected to autoradiography. Finally, the membrane was stripped of radioactivityand reprobed with a *Phaseolus vulgaris H1* probe.


BL + EB (nM)
UV-B + EB (nM)
LW
BL
50
100
200
UVB
50
100
200
CHS
TCH3
H1

4.12 The calmodulin inhibitor, W-7, induces *TCH3* in UV-B light

As mentioned earlier, calmodulin-stimulated Ca^{2+}-ATPases are present at the plasma membrane and intracellular membranes in plant cells. Christie and Jenkins (1996) previously reported that the UV-B induction of *CHS* is sensitive to W-7, suggesting that the UV-B induced reduction of ionophore-stimulated *TCH3* transcript accumulation could involve a CaM-dependent, W-7 sensitive, Ca^{2+}-ATPase. In this case, cells incubated with W-7 prior to addition of ionophore and calcium, and transfer to UV-B light, would be expected to show a full ionophore stimulated *TCH3* response.

Therefore, the effect of pretreatment of cells with W-7 was examined. Figure 4.12 shows that a full *TCH3* response to ionophore was obtained from cells in UV-B light only when cells were pre-incubated with W-7. Full stimulation of *TCH3* in UV-A/blue light was not observed, even in the presence of W-7.

4.13 The calcium channel blocker, nifedipine, does not induce *TCH3* in UV-A/blue or UV-B light

The putative UV/blue light activated Ca^{2+}-ATPase activity appears to be downstream of plasma membrane redox processes. Therefore, it was determined whether the UV-A/blue and UV-B light induced calcium influx via a nifedipine sensitive channel, was upstream or downstream of Ca^{2+}-ATPase activity.

From the result shown in Figure 4.13, it appears that nifedipine did not prevent the proposed UV-A/blue or UV-B light induced decrease in $[Ca^{2+}]_{cyt}$, presumably via Ca^{2+}-ATPase activities. This suggests that activation of the EB and W-7 sensitive pumps is independent of the calcium influx via the nifedipine

Figure 4.12 Effect of W-7 on *TCH3* gene expression in the presence of ionophore

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) without (-) or with 100 μM W-7 for one hour prior to addition of 10 μM A23187 and 10 mM $CaCl_2$ ($+Ca^{2+}$) and transfer to 3 μmol m^{-2} s^{-1} UV-B or 80 μmol m^{-2} s^{-1} UV-A/blue light. Cells were harvested for RNA extraction after 6 hours. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane 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, washed and subjected to autoradiography. Finally, the membrane was stripped of radioactivityand reprobed with a *Phaseolus vulgaris H1* probe.

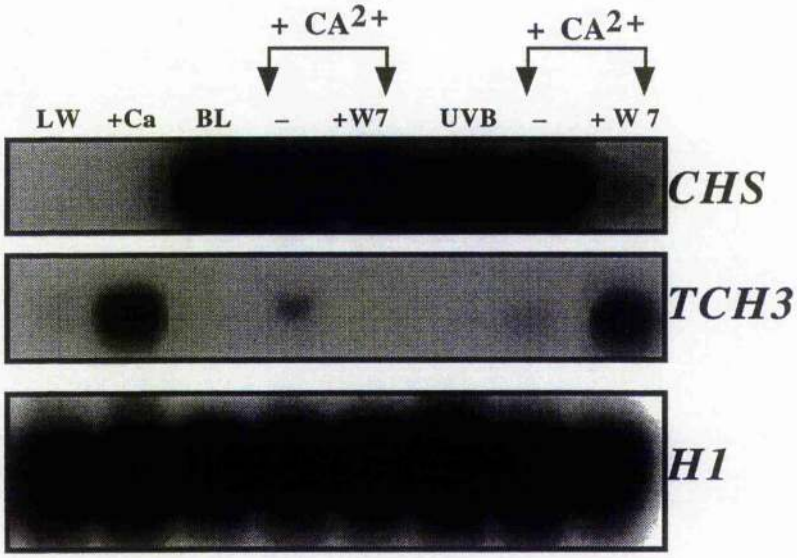
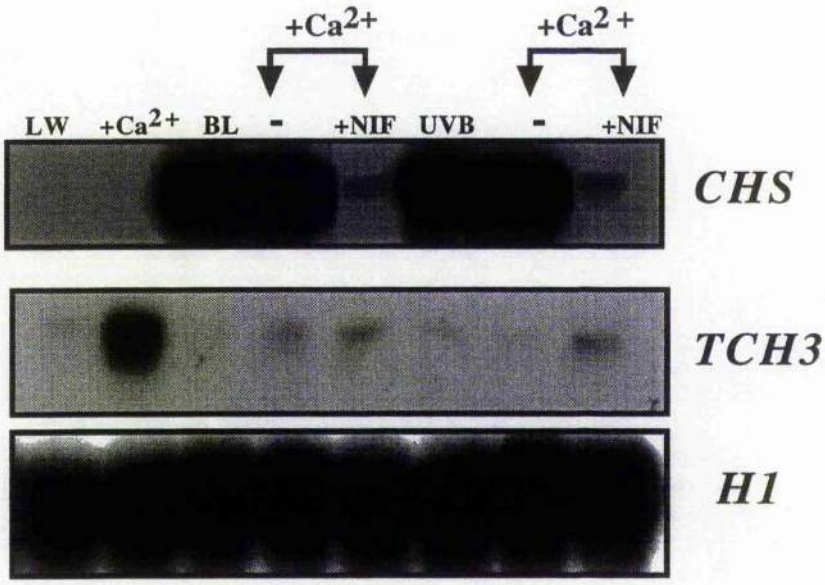

+ CA2+
+ CA2+
LW
+Ca
BL
–
+W7
UVB
–
+ W 7
CHS
TCH3
H1

Figure 4.13 Effect of nifedipine on *TCH3* gene expression in the prescence of ionophore

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) without (BL, UVB) or with 50μM nifedipine (+NIF) for one hour prior to addition of 10 μM A23187 and 10 mM $CaCl_2$ (+ Ca^{2+}), and transfer to 3 μmol m^{-2} s^{-1} UV-B or 80 μmol m^{-2} s^{-1} UV-A/blue light. Cells were harvested for RNA extraction after 6 hours. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane 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, washed and subjected to autoradiography. Finally, the membrane was stripped of radioactivity and reprobed with a *Phaseolus vulgaris H1* probe.


+Ca2+
+Ca2+
LW
+Ca2+
BL
-
+NIF
UVB
-
+NIF
CHS
TCH3
H1

sensitive calcium channel. Hence the nifedipine sensitive calcium influx appears to be downstream of the putative Ca^{2+}-ATPase activities.

4.14 Discussion

In the present chapter, further characterisation of the UV-A/blue and UV-B light signalling pathways regulating *CHS* and *PAL* is described. The effect of artificial electron acceptors was examined in the *Arabidopsis* cell suspension. Plasma membrane redox processes were implicated in the pathways. Preliminary results also suggest that UV/blue light activates a flavoprotein, possibly NADPH oxidase. In addition, the findings led to an investigation of the involvement of P-type Ca^{2+}-ATPases in the UV/blue light signalling pathways. Table 4.1 summarises the main results from this present chapter.

4.15.1 Involvement of plasma membrane electron transport in the UV-A/blue and UV-B light response

The electron acceptors, FeCN and DCPIP, strongly inhibited the UV-A/blue and UV-B light induction of *CHS* and *PAL* gene expression (Figure 4.2). Neither electron acceptor had any significant inhibitory effect on the cantharidin induction of *PAL* transcript accumulation (Figure 4.3A and 4.3B) demonstrating that the compounds are not having a general inhibitory effect on gene expression. Moreover, FeCN actually stimulated *TCH3* expression (Figure 4.2). Although DCPIP may be cell permeable, FeCN is impermeable. This implies that electron transport at the plasma membrane is a necessary component of both the UV-A/blue and UV-B light signalling pathways regulating *CHS* and *PAL*.

Table 4.1 Chapter 4 summary

(A) The table shown is a summary of the experimental treatments described in this present chapter and their effect on *CHS* and *TCH3* transcript accumulation in *Arabidopsis* cells. The UV-A/blue light treatment was 80 μmol m^{-2} s^{-1} and UV-B was 3 μmol m^{-2} s^{-1}.

(B) This table summarizes the effect of treatments described in this present chapter on ionophore induced *TCH3* expression in *Arabidopsis* cells. Cells were pre-treated with calcium ionophore A23187 (10 μM) and $CaCl_2$ (10 mM).

(A)

Treatment	**Effect on UV-A/blue light induced *CHS* expression**	**Effect on UV-B induced *CHS* expression**	**Effect on *TCH3* expression**
500 µM FeCN	Inhibition	Inhibition	Induction
100 µM DCPIP	Inhibition	Inhibition	No Effect
20 µM DPI	Inhibition	Inhibition	Induction
10 µM A23187	No Effect	No Effect	Induction
100 nM EB	Inhibition	No Effect	Induction

(B)

Treatment	**Effect on ionophore mediated induction of *TCH3***
Low white light	Induction
UV-A/blue light	Inhibition
UV-B light	Inhibition
UV-A/blue and 100 µM W-7 pre-treatment	Inhibition
UV-B and 100 µM W-7 pre-treatment	Induction
UV-A/blue and 50 µM nifedipine pre-treatment	Inhibition
UV/blue and 50 µM nifedipine pre-treatment	Inhibition

Blue light has been demonstrated to stimulate redox activity at the plasma membrane of plant cells (Asard *et al.*, 1995; Rubinstein and Luster, 1993; Gautier *et al.*, 1992; Vani and Raghavendra, 1992; Dharmwardhane *et al.*, 1989). The similarity of CRY1 to DNA photolyases suggests that electron transfer could be an initial event in CRY1-mediated signal transduction. Although CRY1 is a soluble protein, it is possible that CRY1 and a putative UV-B photoreceptor could regulate plasma membrane proteins, directly or indirectly.

4.15.2 Induction of *TCH3* expression by FeCN

FeCN, but not DCPIP, was observed to induce transcript accumulation of *TCH3* in low white as well as UV-A/blue and UV-B light (Figure 4.4). Induction was rapid and transient in low white light (Figure 4.5). Although *TCH3* is induced by a number of signals, including mechanical stress and auxin (Braam and Davis, 1990; Braam, 1992; Sistrunk *et al.*, 1994; Braam *et al.*, 1997), these signals also mediate an increase in cytosolic calcium. *TCH3* in *Arabidopsis* cells is induced by artificially increasing cytosolic calcium, using the calcium ionophore A23187 (Braam, 1992; Christie and Jenkins, 1996). It therefore seems likely that FeCN induction of *TCH3* in *Arabidopsis* cells is a reflection of a FeCN induced increase in $[Ca^{2+}]_{cyt}$.

It is well documented that when ferricyanide is reduced to ferrocyanide on the outside of cells or tissues, acidification of the extracellular medium and plasma membrane depoarization occur (Marre *et al.*, 1988; Crane and Barr, 1989). Upon ferricyanide reduction, an extra negative charge arises outside the cells, which is compensated for by the release of H^+ and K^+, so that the total H^+ and K^+ equals electrons transferred by plasma membrane electron transport. The resultant membrane depolarization could gate open plasma membrane voltage-gated calcium channels, leading to an influx of calcium.

The calcium channel blockers, nifedipine and lanthanum, however had no effect on the FeCN induction of *TCH3* (Figure 4.6). This could suggest that calcium influx across the plasma membrane is not involved, and electron transport stimulates calcium release from an internal store which is not inhibited by nifedipine. This could be further investigated by using other calcium channel blockers.

4.15.3 Inhibition of the UV-A/blue and UV-B light responses by DPI

The lipophilic NADPH oxidase inhibitor, DPI, prevented both the UV-A/blue and UV-B light induction of *CHS* and *PAL* (Figure 4.7). The interpretation of these results is that UV/blue light induced plasma membrane redox processes regulate plasma membrane NADPH oxidase activity, resulting in transfer of electrons from intracellular NADPH to molecular oxygen, generation of superoxide, and consequently H_2O_2. One or more of these processes would arguably be involved in the signal transduction pathway regulating *CHS* and *PAL*. DPI did not affect the cantharidin induction of *PAL* (Figure 4.8) which demonstrates that DPI does not have a general detrimental effect on gene expression.

However, although DPI is commonly used in cells to implicate ROS in signalling pathways, DPI also inhibits other redox proteins, including plasma membrane NADPH:quinone oxidoreductase (Trost *et al.*, 1997). DPI was originally used to study mitochondrial electron transport (Doussiere and Vignais, 1992). Additionally, DPI is a lipophilic compound, so the site of action of DPI in these experiments is not clear. The inhibitory action of DPI on mammalian neutrophil NADPH oxidase was recently studied (O'Donnell *et al.*, 1993). DPI was demonstrated to inhibit the flavoprotein component of the enzyme complex, by interfering with electron transfer of the reduced flavin. The result does suggest that

a reduced flavoprotein, and not necessarily NADPH oxidase, is required for both the UV-A/blue and UV-B light signal transduction pathways. DPI could possibly inhibit CRY1 and the UV-B photoreceptor directly, or some downstream electron acceptor. Therefore the role of redox activated ROS is not clear, and is investigated further in Chapter 5.

Interestingly, DPI also rapidly induced *TCH3* expression in low white as well as UV-A/blue and UV-B light (Figure 4.9). This suggests that DPI is also affecting normal ion fluxes, possibly at the plasma membrane. The induction of *TCH3* suggests that that DPI causes an elevation of $[Ca^{2+}]_{cyt}$. However, without knowledge about the site of action of DPI, it is difficult to predict the possible mechanism of calcium entry. The kinetics of *TCH3* induction are also very rapid, and similar to the kinetics of the FeCN induction of *TCH3*.

4.15.4 Role of Ca^{2+}-ATPases in UV-A/blue and UV-B signal transduction

Although both FeCN and DPI induce *TCH3*, probably by altering calcium homeostasis and increasing $[Ca^{2+}]_{cyt}$, treatment of cells with the calcium ionophore did not have any effect on the UV-A/blue or UV-B light induction of *CHS* (Figure 4.10). Therefore, it seems likely that the proposed FeCN and DPI induced calcium influx does not inhibit the UV-A/blue or UV-B signalling pathways. Thus the inhibitory effects of FeCN and DPI on *CHS* expression are likely to be a consequence of the effects on electron transport and not the increased $[Ca^{2+}]_{cyt}$.

Interestingly though, induction of *TCH3* by ionophore/Ca^{2+} was significantly reduced in cells transferred to UV-A/blue or UV-B light for 6 hours (Figure 4.10). This contrasts to the result with either FeCN or DPI (in the absence of ionophore/Ca^{2+}) in which induction of *TCH3* was observed in these light treatments. A possible explanation for these results is that in cells treated with

ionophore the raised $[Ca^{2+}]_{cyt}$ was mobilized out of the cytoplasm by specific transporters activated by the UV-A/blue and UV-B pathways located at the plasma membrane or an internal membrane. In contrast, FeCN and DPI may specifically inhibit the UV/blue light activation of efflux activity. Therefore, activation of a Ca^{2+} efflux transporter may be a component of the UV-A/blue and UV-B signalling pathways. Erythrosin-B, an inhibitor of P-type Ca^{2+}-ATPases, prevents the UV-A/blue but not the UV-B light induction of *CHS* in *Arabidopsis* cells (Figure 4.11). Thus a P-type Ca^{2+}-ATPase may mediate the proposed UV-A/blue light induced Ca^{2+} efflux.

To further investigate the role of UV-A/blue and UV-B light activated Ca^{2+}-ATPases, the assumption was made that UV-A/blue and UV-B light do indeed inhibit ionophore induced *TCH3* transcript accumulation by activating Ca^{2+}-ATPases downstream of plasma membrane redox processes. With this in mind, experiments were carried out to determine whether the UV-B activated efflux is calmodulin-dependent, and also to investigate whether Ca^{2+}-ATPase activity is upstream or downstream of the nifedipine-inhibited calcium channel. Ca^{2+}-ATPase was assumed to be inactive if induction of *TCH3* in UV/blue light was comparable to induction in low white light.

Christie and Jenkins (1996) reported that calmodulin was involved in the UV-B induction of *CHS* transcript accumulation, in contrast to the UV-A/blue induction of *CHS*. *TCH3* transcripts accumulated in UV-B treated cells when the cells were pre-incubated with the calmodulin inhibitor, W-7 (Figure 4.12). Therefore, it appears that UV-B may activate a calmodulin dependent calcium efflux transporter, most likely a Ca^{2+}-ATPase, and this could be the calmodulin dependent component in the UV-B signalling pathway regulating *CHS*. UV-A/blue light activates a separate EB-sensitive Ca^{2+}-ATPase which does not require calmodulin-binding for activation.

Nifedipine did not affect activation of either the UV-A/blue or UV-B light induced putative Ca^{2+}-ATPase activity, as judged by the lack of *TCH3* transcript

accumulation (Figure 4.13). This suggests that activation of the Ca^{2+}-ATPases is downstream of plasma membrane electron transport, but upstream of calcium influx. The significance of this result is discussed further in Chapter 7.

However, the Results discussed above represent indirect pharmacological evidence for a UV-A/blue and UV-B light regulated Ca^{2+}-ATPase. It will be a priority to back up these studies with direct measurements of UV/blue light regulated $[Ca^{2+}]_{cyt}$ fluxes.

CHAPTER 5: INVESTIGATION INTO THE ROLE OF UV/BLUE LIGHT GENERATED REACTIVE OXYGEN SPECIES

5.1 Introduction

The results in the previous chapter implicate plasma membrane redox processes in UV-A/blue and UV-B light induced *CHS* and *PAL* expression. Activation of a plasma membrane superoxide-generating NADPH oxidase was possibly involved. The aim of experiments in this chapter was to look specifically at the role of reactive oxygen species (ROS) in UV-A/blue and UV-B light regulated *CHS* transcript accumulation. Additionally, the effect of UV-A/blue and UV-B light on on expression of three genes encoding proteins involved in protection against oxidative stress was examined.

5.2 H_2O_2 and aminotriazole do not induce *CHS* in non-inductive white light

The intracellular environment of plant cells is maintained in a reduced state. Production of ROS results in an altered intracellular redox status in favour of a more oxidised state, and oxidative stress. Environmental stresses, such as pathogen attack, have been demonstrated to induce oxidative stress in plants, and induction of plant defence genes (Levine *et al.*, 1994). In a less reduced environment, oxidation of critical cysteine residues of redox regulated proteins can occur, resulting in formation of a disulphide bond. Many cytosolic proteins lack disulphide bonds, but oxidation of critical cysteine residues in these proteins results in altered activity, most likely due to a conformational change. Moreover,

transcription factors that are post-transcriptionally regulated by oxidation of critical cysteine residues have been identified in mammalian cells (Wu *et al.*, 1996; Arnone *et al.*, 1995; Nakshatri *et al.*, 1996; Staal *et al.*, 1995). Recently a protein from *Arabidopsis*, ARP was cloned and characterised (Babiychuk *et al.*, 1994). ARP is homologous to a redox-regulated protein from mammalian cells which regulates the DNA-binding activity of transcription factors, Jun and Fos. ARP was demonstrated to regulate the DNA-binding of Fos and Jun (Babiychuk *et al.*, 1994). This represents a mechanism by which ROS could regulate gene expression.

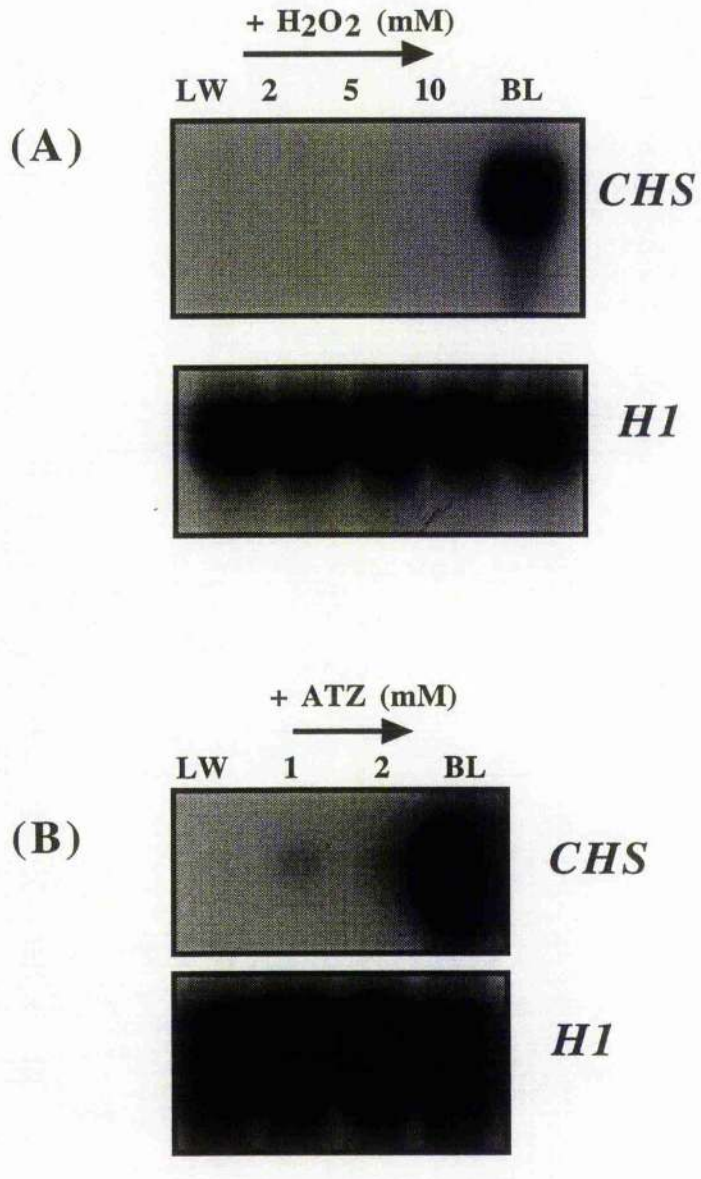
H_2O_2 has been shown to induce plant defence responses, including expression of defense genes (Bi *et al.*, 1995; Chen *et al.*, 1993; Levine *et al.*, 1994; Chen *et al.*, 1996). Therefore, the effect of H_2O_2 in the cell suspension on *CHS* transcript accumulation was examined. As well as the addition of H_2O_2 to the cells, the effect of the catalase inhibitor, 3-amino-1,2,4-triazole (ATZ), was examined. In plant cells, catalases are found in peroxisomes, where they scavenge H_2O_2. Addition of ATZ results in leakage of H_2O_2 from peroxisomes, and has been used to induce oxidative stress in plant cell suspensions (Levine *et al.*, 1994; Bi *et al.*, 1995; May and Leaver, 1993).

Figure 5.1A and 5.1B shows that H_2O_2 and ATZ did not induce *CHS* transcript accumulation in non-inductive white light. This result suggests that H_2O_2 production by UV/blue light is not a component of *CHS* signalling pathways. H_2O_2 and ATZ have been demonstated to induce gene expression in previous studies in intact plants, cell suspension and leaf discs (Bi *et al.*, 1995; Chen *et al.*, 1996; Levine *et al.*, 1994; May and Leaver, 1993). H_2O_2 is a very lipophilic compound, and May and Leaver (1993) previously described catalase inhibition by 2 mM ATZ in *Arabidopsis* cells. It therefore seems likely that the lack of effect by these compounds is due to lack of uptake into the cells.

Figure 5.1 Lack of effect of H_2O_2 and ATZ on *CHS* transcript accumulation in low white light

(A) Cells grown in 20 µmol m^{-2} s^{-1} white light were incubated in 20 µmol m^{-2} s^{-1} white light (LW) in the prescence of H_2O_2 at the concentrations shown or transferred to 80 µmol m^{-2} s^{-1} UV-A/blue light. Cells were harvested for RNA extraction after 6 hours. Equal amounts of RNA (20 µg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane 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 in 20 µmol m^{-2} s^{-1} white light were incubated in 20 µmol m^{-2} s^{-1} white light (LW) in the prescence of ATZ at the concentrations shown or transferred to 80 µmol m^{-2} s^{-1} UV-A/blue light. Cells were harvested for RNA extraction after 6 hours. Transcripts were analyzed as in A.


+ H2O2 (mM)
LW 2 5 10 BL
(A)
CHS
H1
+ ATZ (mM)
LW 1 2 BL
(B)
CHS
H1

5.3 Artificial manipulation of cellular GSH levels does not stimulate *CHS* transcript accumulation

H_2O_2 and O_2^- in the presence of metal ions will form the highyl reactive hydroxyl radical, OH^-, one of the most reactive compounds known in chemistry. OH^- can cause extensive damage in biological systems, reacting with proteins, lipids and nucleic acids, ultimately leading to cell death. As OH^- is so reactive, it would be difficult for the plant cell to protect itself. Therefore protective mechanisms to avoid OH^- production, by preventing accumulation of H_2O_2 and O_2^-, are present in plant cells. These protective mechanisms consist of enzymic and non-enzymic components (Foyer *et al*, 1994; Foyer *et al*, 1997; Alscher *et al*, 1997). Particularly important in the protection against ROS is the small molecular weight thiol, glutathione, which occurs in the oxidised form (GSSG) and the reduced form (GSH). Oxidative stress has been shown to stimulate glutathione synthesis in *Arabidopsis* cells (May and Leaver, 1993). Several studies have demonstrated a role for glutathione in ROS mediated gene expression. Addition of GSH, but not GSSG, to plant cells has been demonstrated to stimulate gene expression (Herouart *et al.*, 1993; Wingsle *et al.*, 1996), including *CHS* and *PAL* transcripts in *Phaseolus vulgaris* cell suspension (Wingate *et al.*, 1988).

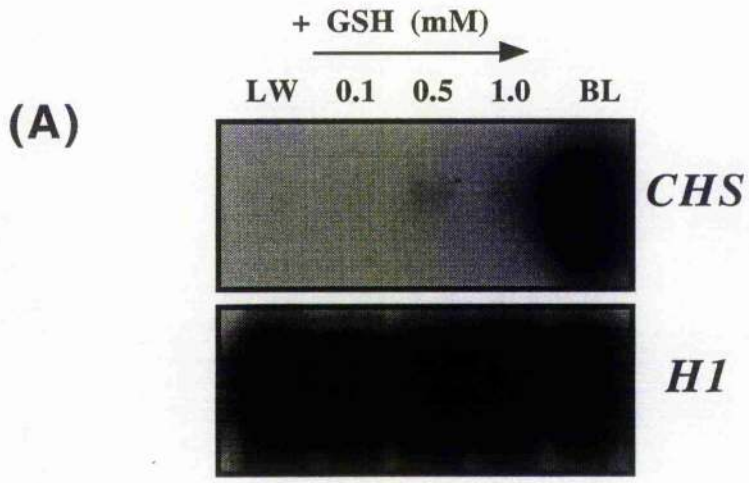
Therefore, the effect of artificially altering the glutathione content of *Arabidopsis* cells on *CHS* transcript levels was examined. As shown in Figure 5.2A, GSH did not stimulate *CHS* transcript accumulation in non-inductive white light. Wingate *et al.* (1988) used 1 mM GSH to induce gene expression in a *Phaseolus vulgaris* cell suspension, indicating that GSH is taken up into cells.

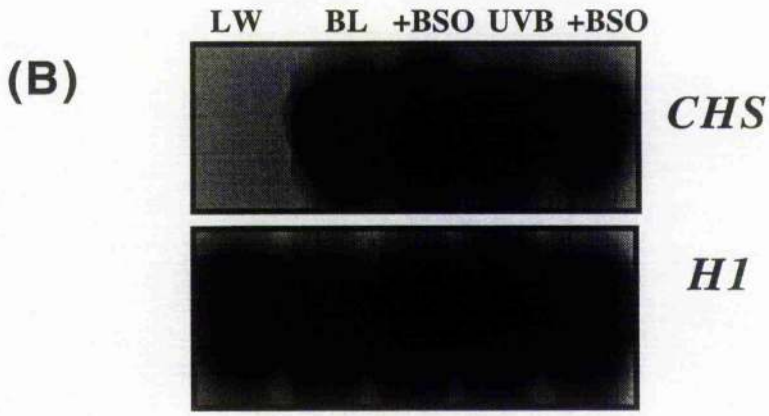
Additionally, a pharmacological inhibitor of glutathione synthesis, buthionine sulfoximine (BSO), has been demonstrated to reduce cellular glutathione levels by specifically inhibiting γ-glutamyl-cysteine-synthetase (Price *et al.*, 1994; May and Leaver, 1993). However, BSO had no affect on UV-A/blue or UV-B light induced *CHS* expression (Figure 5.2 B). 50 μM BSO was found to

Figure 5.2 Lack of effect of GSH and BSO on *CHS* transcript accumulation in low white, UV-A/blue and UV-B light

(A) Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) in the presence of GSH. Cells were harvested for RNA extraction after 6 hours. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane 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 in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) in the presence of 50 μM BSO for one hour prior to transfer to 80 μmol m^{-2} s^{-1} UV-A/blue or 3 μmol m^{-2} s^{-1} UV-B light. Cells were harvested for RNA extraction after 6 hours. Transcripts were analysed as in A.


(A)
+ GSH (mM)
LW
0.1
0.5
1.0
BL
CHS
H1


(B)
LW
BL
+BSO
UVB
+BSO
CHS
H1

be effective in *Arabidopsis* suspension cells (May and Leaver, 1993), preventing glutathione accumulation in response to oxidative stress. This suggests that the lack of effect is not due to lack of uptake an ineffective concentration of BSO. These results suggest that glutathione synthesis is not a component in UV/blue light signalling.

5.4 Active oxygen species scavengers, NAC and PDTC, do not inhibit *CHS* expression in UV-A/blue or UV-B light

Recently, Green and Fluhr (1995) reported that the active oxygen scavengers, N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) prevented the UV-B induced expression of the pathogenesis related gene, *PR-1* in tobacco. This suggested that ROS generated by UV-B light stimulated *PR-1* expression.

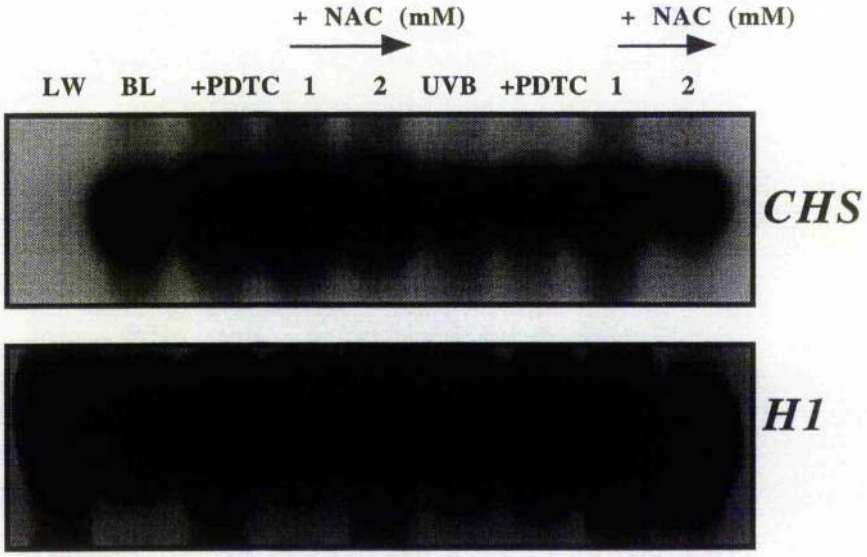
Therefore the effect of NAC and PDTC on UV-B and UV-A/blue light induced *CHS* transcript accumulation in *Arabidopsis* cells was determined. As can be seen in Figure 5.3, neither scavenger had any effect on *CHS* expression in either light condition. Up to 2 mM NAC was used, which was the highest concentration that was not toxic to the cell culture. Also, 100 μM PDTC had no effect, which was the same concentration Green and Fluhr (1995) used to spray onto tobacco plants. Therefore it seems likely that the lack of effect is not due to lack of uptake of the compounds into the cells.

5.5 H_2O_2 has no effect on *GST5* or *APX1* in the cell culture environment

The above results suggest that UV-A/blue and UV-B light induced *CHS* expression in the *Arabidopsis* cell culture does not involve ROS. However, it was

Figure 5.3 Effect of active oxygen scavengers on *CHS* transcript accumulation in UV-B and UV-A/blue light

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light (LW) without (BL, UVB) or with 100 μm PDTC or NAC at the concentrations shown for one hour prior to transfer to 3 μmol m^{-2} s^{-1} UV-B or 80 μmol m^{-2} s^{-1} UV-A/blue light. Cells were harvested for RNA extraction after 6 hours. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane 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.


+ NAC (mM)
+ NAC (mM)
LW
BL
+PDTC
1
2
UVB
+PDTC
1
2
CHS
H1

important to demonstrate that the compounds used in this study were effective in altering cellular redox status. Therefore, their effects on the induction of control gene expression was investigated. Hence the aim of the experiments described in the remainder of this chapter was to identify an appropriate control. The effect of compounds, described previously, on three commonly studied genes involved in plant protection was examined.

The antioxidant enzyme, ascorbate peroxidase (APX), along with other peroxidases and catalases, scavenges H_2O_2 (Foyer *et al.*, 1994). APX catalyzes oxidation of the important cellular antioxidant ascorbate, which is subsequenly regenerated by enzymes of the Halliwell-Asada pathway. APX in plant cells is found both in the chloroplast and cytosol (Bowler *et al.*, 1992). APX transcript accumulation is increased by environmental stresses which induce oxidative stress in plants, including ozone (Conklin and Last, 1995; Kubo *et al.*, 1995), sulphur dioxide (Kubo *et al.*, 1995) and excess light (Karpinski *et al.*, 1997). The *APX1* gene from *Arabidopsis* encodes a cytosolic ascorbate peroxidase (Kubo *et al.*, 1992). A 500 bp gene-specific probe generated from the *APX1* sequence by PCR amplification (Karpinski *et al.*, 1997) was donated by Dr. P. Mullineaux (John Innes Centre, Norwich, UK).

After an initial pathogen attack, plants become resistant to a subsequent attack, a phenomenon termed systemic acquired resistance (SAR) (Ryals *et al.*, 1996; Hunt *et al.*, 1996). Certain natural chemicals can also induce SAR in plants, including salicylic acid (SA) (Vernooij *et al.*, 1994). Associated with SAR is the induction of a subset of genes called the *SAR* genes. Five of these gene families encode the pathogenesis-related (PR) proteins, extracellular polypeptides associated with pathogen infection whose physiological functions have yet to be determined (Uknes *et al.*, 1992). It has been demonstrated that SA binds to a catalase in tobacco cells, inhibiting catalase activity (Chen *et al.*, 1993). However, although SA has been demonstrated to inhibit catalases, the mode of action of SA is still the subject of debate, in particular its role in H_2O_2 accumulation and *PR-1*

expression (Durner and Klessig, 1996; Bi *et al.*, 1995). This is currently an area of intense research. UV-B light has also been shown to induce *PR-1* expression in tobacco leaves, and this induction of *PR-1* was inhibited by active oxygen scavengers, suggesting that UV-B light-induced *PR-1* expression was mediated via ROS (Green and Fluhr, 1995). However, UV-B light was shown not to induce *PR-1* transcript accumulation in tomato (Conconci *et al.*, 1996). Plasmid p*PR-1*, containing the *Arabidopsis PR-1* cDNA (Uknes *et al.*, 1992) fragment was donated by Dr. J. Ryals (Glaxo-Wellcome, NC, USA).

The glutathione-S-transferase (*GST*) enzymes catalyze the conjugation of GSH to hydrophobic, electrophilic and cytotoxic substrates (Marrs, 1996). GS-conjugates are subsequently recognized by ATP-dependent glutathione pumps at plasma and vacuolar membranes, and either sequestered to the vacuole or transferred to the apoplast. GSTs play an important role in protection against oxidative damage, and soybean *GST* gene expression has been shown to be induced by H_2O_2 (Levine *et al.*, 1994). *Arabidopsis GST6* expression is also induced by H_2O_2 in *Arabidopsis* plants (Chen *et al.*, 1996). The cDNA for an *Arabidopsis GST5* isoform was recently reported (Watahiki *et al.*, 1995) and primers for the *GST5* isoform, designed to span an intron in the genomic clone were donated by Dr. M.R. Knight (Oxford University, UK). PCR was carried out with *Arabidopsis* DNA and the 500 bp fragment was obtained (Figure 5.4).

The effect of H_2O_2 on these 3 protective genes was examined. As can be seen in Figure 5.5, 10 mM H_2O_2 appeared to slightly induce *APX1*, but no effect on *GST5* or *PAL* was observed. *APX1* and *GST5* transcripts were present in cells in low white light without addition of H_2O_2. No induction of *PR-1* was detected in any treatment (data not shown), suggesting that *PR-1* may not be induced in cell suspensions. As described earlier, no induction of *CHS* transcripts was observed.

Figure 5.4 PCR amplifiacation of *GST5* in *Arabidopsis*

Primers (5'-TTGCTTCTTGCTCTTAACCC-3') and (5'-GGAATTTGGAGAAG GTTGAG-3') designed to span an intron in the *GST5* genomic clone (Watahiki *et al.*, 1995) were kindly donated by Dr. M.R. Knight (Oxford University, Oxford). PCR was performed as described in Section 2.9.2. A control reaction without *Arabidopsis* genomic DNA was also performed. After PCR, 2 µl of the following were separated on a 1% agar gel: (1) 1 kb ladder (Promega), (2) no DNA control and (3) PCR reaction with genomic DNA.

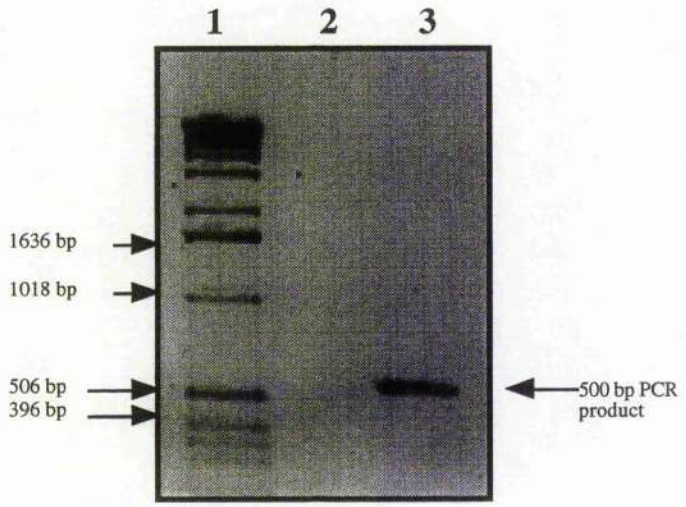
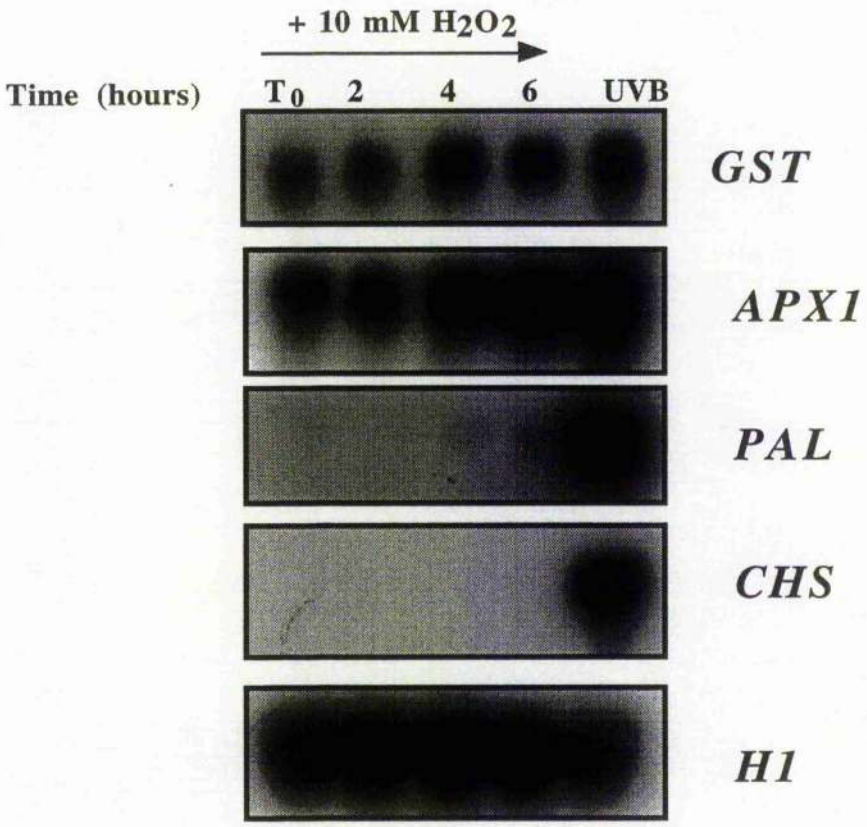

1
2
3
1636 bp
1018 bp
506 bp
396 bp
500 bp PCR
product

Figure 5.5 Effect of H_2O_2 on *GST*, *APX1*, *CHS* and *PAL* transcript accumulation in low white light

Cells grown in 20 μmol m^{-2} s^{-1} white light were incubated in 20 μmol m^{-2} s^{-1} white light without (T_0) or with 10 mM H_2O_2 for the times indicated or transferred to 3 μmol m^{-2} s^{-1} UV-B light (UVB). After 6 hours cells were harvested for RNA extraction. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with an *Arabidopsis GST* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis APX1* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. The membrane was stripped of radioactivity and reprobed with an *Arabidopsis CHS* probe, washed and subjected to autoradiography. Finally, the membrane was stripped and reprobed with a *Phaseolus vulgaris H1* probe.


+ 10 mM H_2O_2
Time (hours)
T_0 2 4 6 UVB
GST
APX1
PAL
CHS
H1

5.6 Reduced glutathione, aminotriazole and SA does not have an effect on *GST5* or *APX1* gene expression

The compounds, ATZ, SA and GSH, described above, have previously been shown to induce *GST* in soybean cells (Shirasu *et al.*, 1997; Levine *et al.*, 1994). Therefore, the effect of these compounds on the accumulation of *GST5* and *APX1* transcripts in *Arabidopsis* cells was examined. Figure 5.6 shows that transcripts for *APX1* and *GST5* were abundantly expressed, and did not increase above the levels observed in the non-treated control sample with any of the compounds.

5.7 UV-A/blue and UV-B light induce *GST5* but not *APX1* in the cell culture

There is accumulating evidence that UV-B light induces oxidative stress responses in *Arabidopsis* (Landry *et al.*, 1995; Rao *et al.*, 1996). Additionally, induction of defence and antioxidant genes has been proposed to be mediated by ROS (Strid, 1993; Green and Fluhr, 1995; Willekens *et al.*, 1994).

Therefore, the effect of UV-A/blue and UV-B light on *GST5* and *APX1* transcripts in *Arabidopsis* cells was examined. Figure 5.7 shows that both *GST5* and *APX1* transcripts were detectable in the control low white light treatment. *APX1*, however, was not increased by UV-A/blue or UV-B light. Willekens *et al.* (1994) also reported that *cytAPX* is not induced by UV-B light. However, UV-A/blue and UV-B strongly induced *GST5* expression in the cell culture. It would therefore be interesting to determine whether the ROS scavengers, NAC and PDTC, which have no effect on UV-A/blue and UV-B induction of *CHS*, inhibit UV/blue light induction of *GST*.

Figure 5.6 Effect of ATZ, GSH and SA on *GST* and *APX1* transcript accumulation in low white light

Cells grown in 20 μmol m^{-2} s^{-1} white light were harvested (T_0) or incubated in 20 μmol m^{-2} s^{-1} white light with 2 mM SA, 2 mM ATZ or 2 mM GSH for the times indicated. After the stated time, cells were harvested for RNA extraction. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with an *Arabidopsis GST* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis APX1* probe, washed and subjected to autoradiography. Finally, the membrane was stripped and reprobed with a *Phaseolus vulgaris H1* probe.

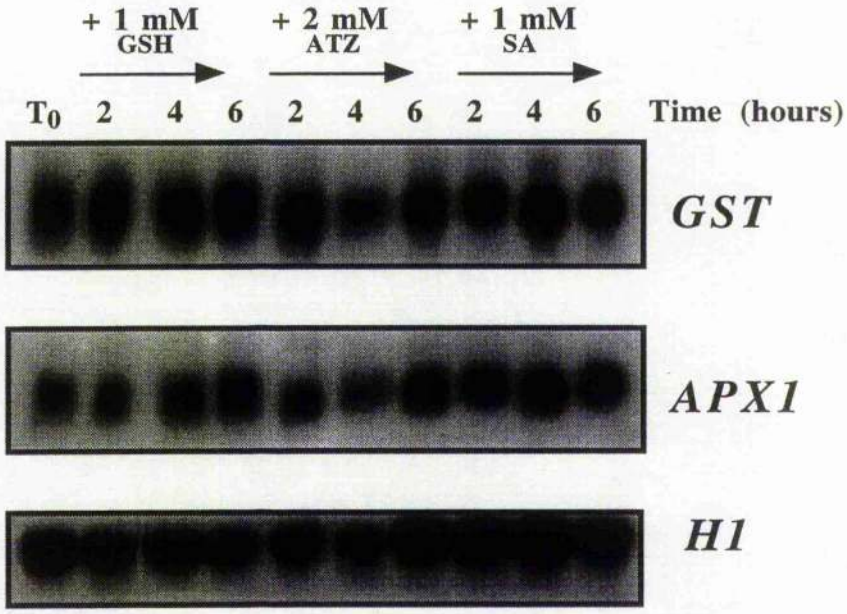
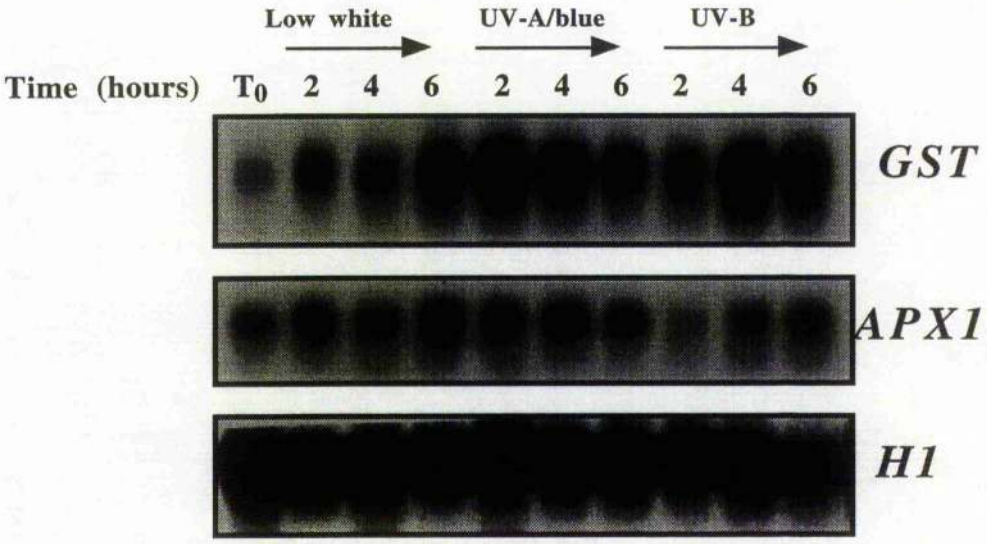

+ 1 mM
GSH
+ 2 mM
ATZ
+ 1 mM
SA
T0 2 4 6 2 4 6 2 4 6 Time (hours)
GST
APX1
H1

Figure 5.7 Effect of UV-B and UV-A/blue light on *GST* and *APX1* transcript accumulation

Cells grown in 20 μmol m^{-2} s^{-1} white light were harvested (T_0) or incubated in 20 μmol m^{-2} s^{-1} white (LW), 3 μmol m^{-2} s^{-1} UV-B (UVB) or 80 μmol m^{-2} s^{-1} UV-A/blue light (B) for the times indicated. After the stated time, cells were harvested for RNA extraction. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with an *Arabidopsis GST* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis APX1* probe, washed and subjected to autoradiography. Finally, the membrane was stripped and reprobed with a *Phaseolus vulgaris H1* probe.


Low white
UV-A/blue
UV-B
Time (hours) T0 2 4 6 2 4 6 2 4 6
GST
APX1
H1

5.8 UV-A/blue and UV-B light induce *GST5* but not *PR-1*, and salicylic acid induces *PR-1* but not *GST5*, in *Arabidopsis* plants

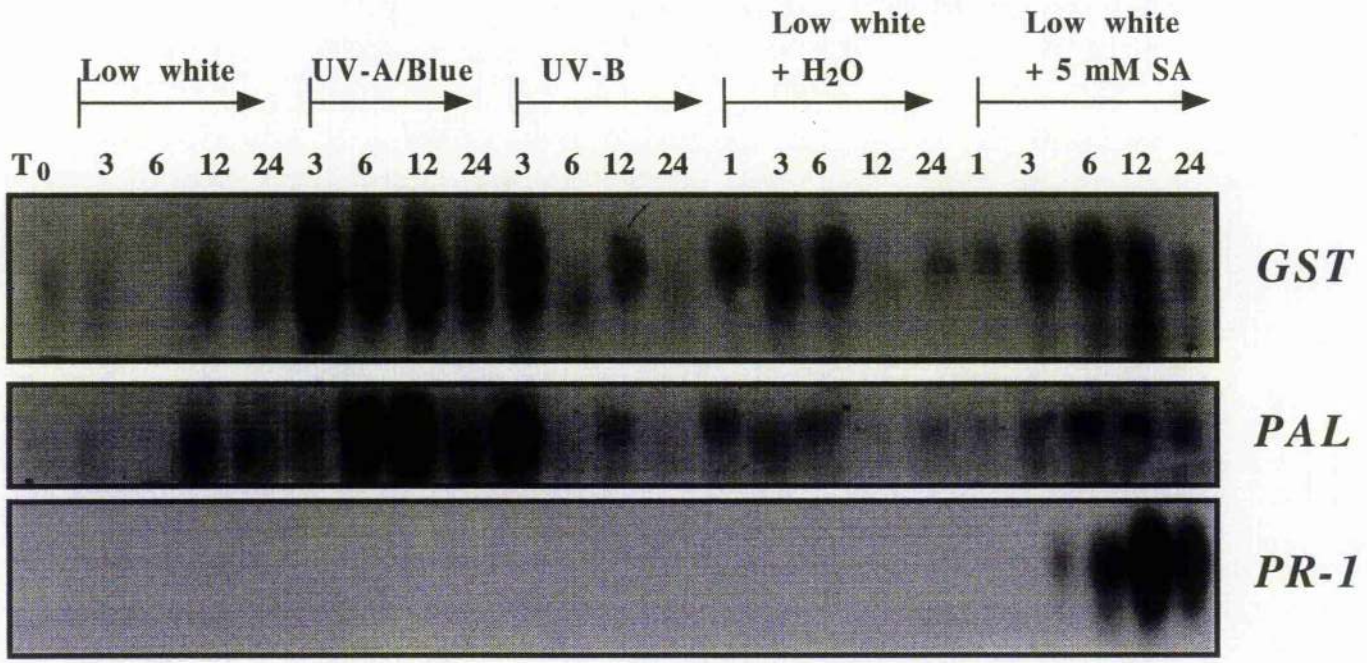
Although UV-A/blue and UV-B light strongly increased *GST5* transcript accumulation in the cells, the results presented earlier demonstrate that compounds used to increase the oxidative status of *Arabidopsis* cells had no effect on *GST5* or *APX1* gene expression. In *Arabidopsis* plants, *GST5* transcripts, barely detectable by northern analysis, were massively induced by mechanical injury (Watahiki *et al.*, 1995). This suggests that mechanical stimulation of the *Arabidopsis* cells by shaking may be inducing *GST5* and *APX1* transcripts in low white light. Thus, further stimulation in response to oxidative stress induced by various compounds may not be detectable. Mechanical shaking may in fact induce a constituitive oxidative stress response, or the cells may be de-sensitised to oxidative stress.

No expression of *PR-1* was ever detected in any light or SA treatments of the cell culture (data not shown), which suggests that *PR-1* is not induced in the cell suspension environment. Therefore, we decided that the *Arabidopsis* cell culture may not be an appropriate model system to study regulation of these genes. For these reasons, preliminary experiments using *Arabidopsis* plants were carried out. The aim of these experiments was simply to determine whether UV-A/blue and UV-B light also induce *GST5* transcripts in *Arabidopsis* plants. In addition, the effect of UV-A/blue and UV-B light on *PR-1* expression was examined, using SA as a control.

Figure 5.8 shows that *GST5* transcripts were induced by UV-A/blue and UV-B light within 3 hours in a similar manner to the cell suspension. However, neither UV-A/blue or UV-B light had any effect on *PR-1* transcripts. In contrast,

Figure 5.8 Effect of UV-B, UV-A/blue light and SA on *GST*, *PAL* and *PR-1* transcript accumulation in *Arabidopsis* plants

Plants grown in 20 μmol m^{-2} s^{-1} white light for 21 days were transferred to 20 μmol m^{-2} s^{-1} white, 80 μmol m^{-2} s^{-1} UV-A/blue light or 3 μmol m^{-2} s^{-1} UV-B light, or sprayed with tap water or 5 mM SA in 20 μmol m^{-2} s^{-1} white light. Samples were harvested for RNA extraction at the time indicated. Equal amounts of RNA (20 μg) were separated on a 1.3 % denaturing agarose gel and blotted on to a nylon membrane. The membrane was probed with an *Arabidopsis GST* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis PAL* probe, washed and subjected to autoradiography. The filter was stripped and reprobed with an *Arabidopsis PR-1* probe, washed and subjected to autoradiography.


Low white
UV-A/Blue
UV-B
Low white
+ H2O
Low white
+ 5 mM SA
T0 3 6 12 24 3 6 12 24 3 6 12 24 1 3 6 12 24 1 3 6 12 24
GST
PAL
PR-1

spraying plants with SA, but not water, strongly induced *PR-1* expression. *PAL* expression was also induced by SA in a similar kinetic manner to *PR-1*, although transcript levels were not as strongly increased. *GST5* transcipt accumulation was increased by spraying plants with both water and SA, although to a lesser extent than with UV-A/blue or UV-B light.

5.9 Discussion

In the present chapter, the role of ROS in UV-A/blue and UV-B induced *CHS* expression was investigated using a biochemical approach in the *Arabidopsis* cell culture. It was demonstrated that the addition of compounds which induce or inhibit oxidative stress had no effect on *CHS* transcript accumulation, suggesting that UV/blue light regulated plasma membrane redox activity does not generate ROS. Table 5.1 summarises the main results from this present chapter. Additionally, a possible control response was identified for future studies.

5.9.1 Involvement of ROS in UV-A/blue and UV-B light signalling

Oxidative stress can be defined as the production of ROS and resulting in an alteration in the cellular redox status in favour of a more oxidised state. A mechanism for oxidative stress regulation of gene expression by redox regulation of transcription factor DNA-binding activity, has been proposed in mammalian signalling pathways (Wu *et al.*, 1996; Arnone *et al.*, 1995; Nakshatri *et al.*, 1996; Staal *et al.*, 1995). UV-B light has been proposed to induce oxidative stress as a signal component of plant gene expression (Strid, 1993; Green and Fluhr, 1995; Willekens *et al.*, 1994). Although there is less information on the role of UV-A/blue light in oxidative stress, blue light regulated plasma membrane redox

Table 5.1 Chapter 5 summary

The table shown is a summary of the experimental treatments described in this present chapter and their effect on *CHS* transcript accumulation in *Arabidopsis* cells. The light treatments used were low white light (20 μmol m^{-2} s^{-1}), UV-A/blue light (80 μmol m^{-2} s^{-1}) and UV-B (3 μmol m^{-2} s^{-1}).

Treatment	**Effect on *CHS* expression in low white light**	**Effect on *CHS* expression in UV-A/blue or UV-B light**
10 mM H2O2	No Effect	Not determined
2 mM ATZ	No Effect	Not determined
1 mM GSH	No effect	Not determined
50 µM BSO	Not determined	No effect
100 µM PDTC	Not determined	No effect
2 mM NAC	Not determined	No effect

activity has been proposed to generate production of ROS by electron transfer to molecular oxygen (Asard *et al.*, 1995). The plasma membrane of plant cells contains several oxidoreductases (Rubinstein and Luster, 1993). The results described in Chapter 4 demonstrated that redox activity at the plasma membrane is probably an important component of UV/blue light regulated *CHS* and *PAL* gene expression in *Arabidopsis* cells. Additionally, the inhibition of UV-A/blue and UV-B induced *CHS* expression by DPI raised the possibility that a plasma membrane superoxide-generating oxidase may be involved. Therefore, it was of interest to investigate whether ROS are involved in mediating UV-A/blue or UV-B induced *CHS* expression in *Arabidopsis.*

UV-A/blue or UV-B light activation of the superoxide-generating plasma membrane NADPH oxidase would be expected to generate O_2^- and ultimately H_2O_2 outside the cell. H_2O_2 is a lipophilic signalling molecule which regulates expression of plant defence genes (Bi *et al.*, 1995; Chen *et al.*, 1993; Levine *et al.*, 1994; Chen *et al.*, 1996). We therefore investigated the effect of H_2O_2 on *CHS* transcript accumulation.

H_2O_2 did not induce *CHS* transcript accumulation in non-inductive white light (Figure 5.1A). The concentrations used in these experiments are within the range used to induce gene expression in other studies. Additionally the cell permeable catalase inhibitor, ATZ, did not induce *CHS* transcripts (Figure 5.1B). Together, these findings suggest that the generation of H_2O_2 is not involved in UV/blue light signalling pathways regulating *CHS* expression. Since DPI also inhibits other flavoproteins, it seems likely that the effect of DPI was not due to specific inhibition of the superoxide-generating NADPH oxidase and that some other flavoprotein involved in UV/blue light mediated electron transport was the target of inhibition.

Glutathione is an important antioxidant in plants, and plays a vital role in maintaining the reduced intracellular environment. Additionally, glutathione synthesis in response to oxidative stress has been proposed to regulate gene

expression (Wingate, 1988; Wingslc, 1996; Herouart, 1993). However, addition of GSH to *Arabidopsis* cells in non-inducive white light had no effect on *CHS* transcript accumulation (Figure 5.2A). Additionally, the glutathione synthesis inhibitor, BSO, did not inhibit UV-A/blue or UV-B light induced *CHS* expression (Figure 5.2B). Both GSH and BSO have been used previously to affect gene expression at the concentrations employed in this study in cell suspensions and intact tissue (Levine *et al.*, 1994; Chen *et al.*, 1996; Bi *et al.*, 1995; Wingate *et al.*, 1988; May and Leaver, 1993).

The ROS scavengers, NAC and PDTC, did not have any effect on either the UV-A/blue or UV-B light induction of *CHS*. It is likely that the lack of inhibition is not due to lack of uptake of the compounds into the cells as NAC is toxic to *Arabidopsis* cells at concentrations greater than those shown in Figure 5.3. PDTC was used at the same concentration as Green and Fluhr (1995) applied to tobacco leaves. Therefore, UV-A/blue and UV-B light regulated *CHS* expression in *Arabidopsis* cells does not appear to be mediated by ROS. This suggests that plasma membrane redox activity does not cause reduction of oxygen as an essential component of the signalling pathway regulating *CHS* expression.

It would now be valuable to complement the experiments discussed above with direct measurements of H_2O_2 and O_2^- production following UV-A/blue and UV-B light. It remains possible that ROS could be involved in the regulation of other genes by UV/blue light.

5.9.2 Regulation of protective genes in *Arabidopsis* cells

Experiments were undertaken to identify a gene which is upregulated by oxidative stress in *Arabidopsis* cells. It would then be possible to examine the effects of compounds used in this study, which had no effect on UV/blue light induction of *CHS*, on a control response. This is important, as it is necessary to be sure that

the results described above were not due to lack of activity of the compounds in the *Arabidopsis* cells. Oxidative stress has been shown to regulate expression of genes involved in protective mechanisms in plant cells. Therefore the regulation of three such genes in the cell culture was examined.

In all the experiments with the cell culture, *PR-1* transcripts were not detected (data not shown). However, this was not due to experimental problems with the cDNA probe used, as *PR-1* transcripts were detected at a detectable level in *Arabidopsis* plants (Figure 5.8). This suggests that the *PR-1* gene is not expressed in the *Arabidopsis* cell culture environment.

Transcripts for *GST5* and *APX1*, however, were abundantly expressed in all treatments, including the non-inductive white light control, and appeared to increase during the experimental treatment in non-inductive light (Figure 5.7). *APX1* is detected in *Arabidopsis* under the same fluence rate (Karpinski *et al.*, 1997), but *GST5* was reported to be barely detectable in de-etiolated *Arabidopsis* plants which had been transferred to darkness. However, *GST5* transcripts were massively induced in *Arabidopsis* plants by mechanical injury and spraying buffer onto the plants, and it was proposed that mechanical stimulation and injury could induce oxidative stress and consequently *GST5* expression (Watahiki, 1995). It is therefore possible that the observed levels of *GST5* and *APX1* transcripts in *Arabidopsis* cells could be due to mechanical stimulation of cells during the experiment. Neither H_2O_2, ATZ, SA or GSH increased transcript accumulation of *GST5* or *APX1* in the cell culture. This may be because the cells had become desensitised to the effects of these compounds, because of the level of oxidative stress caused by mechanical shaking.

APX1 transcripts were not increased by either UV-A/blue or UV-B light. *GST5* expression, however, was stongly increased upon illumination with UV-A/blue or UV-B light. This is an interesting result, and is discussed further below.

Several results suggested that the *Arabidopsis* cell culture was not suitable for studying the oxidative regulation of the chosen genes. Therefore, we decided to continue the studies in *Arabidopsis* plants.

5.9.3 Regulation of protective genes in *Arabidopsis* plants

Preliminary experiments were carried out in *Arabidopsis* plants to examine regulation of the *GST5* and *PR-1* genes. As *APX1* was not induced in *Arabidopsis* cells by any treatment, it was decided not to pursue regulation of this gene any further.

In this study, it was shown that neither UV-A/blue or UV-B light induced *PR-1* transcripts in *Arabidopsis* plants. Moreover, Conconchi *et al.* (1996) reported that UV-B light did not induce *PR-1* in tomato. Additionally, Dr. H. Frohnmeyer (Freiburg, Germany) found that UV-B did not induce *PR-1* in parsley plants (personal communication). *PR-1* is induced in response to pathogen attack and is associated with SAR. Although the physiological function of PR proteins is not known, PR proteins have been proposed to play a role in the resistance state after an initial pathogen attack (Uknes *et al.*, 1992). Therefore it is probably not unexpected that UV-A/blue and UV-B light do not induce the accumulation of pathogen resistance proteins. The UV-B induction of *PR-1* in tobacco (Green and Fluhr, 1995) could be an observation of species difference.

In the experiment shown in Figure 5.8, plants were treated with SA as a control. *PR-1* was strongly induced by 5 mM SA in *Arabidopsis* plants, as reported by Uknes *et al.*., (1992). SA also slightly induced *PAL* transcripts in a similar kinetic manner. *GST5* transcripts were increased by spraying plants with either water or SA. Induction of *GST5* in *Arabidopsis* plants was induced by spraying *Arabidopsis* plants with buffer (Watahiki *et al.*, 1995), and it was suggested that spraying plants with buffer mechanically stimulated *GST5* in a similar manner to the touch induced (*TCH*) genes (Braam and Davis, 1990;

Braam, 1992; Braam, 1997). This mechanical stimulation response may be mediated by ROS. SA, however, did not induce *GST5* above levels observed for the water control. Notably, transcipt abundance of *GST5* in non-inductive low white light was much lower in *Arabidopsis* plants than in cell culture. This is consistent with the suggestion that shaking of the cell culture induced *GST5* expression.

GST5 transcripts were strongly induced by UV-A/blue and UV-B light in plants in a similar manner to the cell culture. As GSTs are involved not only in protection against oxidative stress in plant cells, but also in normal cell processes (Marrs *et al.*, 1996) it is not clear whether the UV-A/blue and UV-B induction of *GST5* transcripts was a result of increased oxidative stress. The lack of induction of *GST5* by SA above the level observed for the water control suggests that *GST5* induction by UV-A/blue and UV-B could be independent of ROS.

Interestingly, GSTs play an important role in anthocyanin biosynthesis. Anthocyanins are targeted to the vacuole by glutathione conjugation, and subsequent recognition by a vacuolar GST pump (Marrs, 1996). The maize gene, *bronze-2*, encodes a GST that catalyzes the formation of anthocyanin-GSH conjugates, and *bronze-2* mutants accumulate the anthocyanin precursor, cyanidin-3-glucoside in the cytoplasm, causing localized necrosis, poor vigor and even death (Marrs *et al.*, 1995). Additionally GST is active in *Arabidopsis*. The anthocyanin deficient mutant, *ttg*, displays reduced GST activity compared to wild-type, and this activity is increased by transformation of *ttg* with *bronze-2* (Marrs *et al.*, 1995). It would be interesting to determine whether *GST5* mRNA is increased by UV-A/blue and UV-B light as a response to increased anthocyanin in the plant cell.

5.9.4 Identification of suitable controls for oxidative stress experiments

The results in this section suggest that ROS are not involved in UV/blue light. However, the identification of controls has been carried out. SA induction of *PR-1* transcripts in *Arabidopsis* plants has been reported previously (Uknes *et al.*, 1992). In this study we observed that *PAL* transcripts were also induced by SA in *Arabidopsis* plants. Assuming that SA induces oxidative stress by increasing H_2O_2 in the cell, experiments to determine whether this treatment also increases *CHS* transcripts in *Arabidopsis* plants needs to be carried out. Currently, the data suggest that the observed UV-A/blue and UV-B light induction of *GST5* in *Arabidopsis* plants is not mediated by ROS. It would be interesting to determine the effect of the ROS scavengers, NAC and PDTC, which have no effect on UV/blue light regulated *CHS* expression, on UV/blue induced *GST5* transcript accumulation. Unfortunately, due to time constraints it was not possible to continue these experiments for this thesis.

CHAPTER 6: UV-A/BLUE LIGHT INDUCED CALCIUM SIGNALLING

6.1 Introduction

Biochemical analysis of UV-A/ blue and UV-B light regulated gene expression suggest that Ca^{2+} plays an important role in the signalling pathways. Clearly it is essential to test this hypothesis by direct measurements of UV/blue induced changes in cytosolic calcium concaentrations ($[Ca^{2+}]_{cyt}$). The experiments reported in this chapter examined the effect of UV-A/blue light on $[Ca^{2+}]_{cyt}$ Measurements of UV-B induced effects on $[Ca^{2+}]_{cyt}$ were more difficult technically and were not attempted. To measure $[Ca^{2+}]_{cyt}$, transgenic *Arabidopsis* wild-type and *hy4* plants were generated which expressed cytoplasmic apoaequorin. A UV-A/blue light induced $[Ca^{2+}]_{cyt}$ increase was characterised in *Arabidopsis* and *hy4*, and the effect of pharmacological Ca^{2+} inhibitors on the response was determined.

6.2 Production of transgenic plants

A method of measuring $[Ca^{2+}]_{cyt}$ in plant cells was described by Knight *et al.*, (1991). Tobacco plants were genetically engineered to express apoaequorin, the jellyfish (*Aequoria victorea*) protein which, when bound to its co-factor, coelenterazine, emits photons at a rate dependent on $[Ca^{2+}]_{cyt}$. Genetically transformed tobacco and *Arabidopsis* plants expressing cytosolic aequorin have been used to measure Ca^{2+} increases in response to a variety of environmental signals including cold-shock (Knight *et al.*, 1991, 1996), circadian oscillations (Johnson *et al.*, 1995), anoxia

(Sedbrook *et al.*, 1996), mechanical stimulation (Knight *et al.*, 1992; Knight *et al.*, 1995), hypoosmotic shock (Takahashi *et al.*, 1997) and oxidative stress (Price *et al.*, 1994).

Results from this study and other studies (Christie and Jenkins, 1996; Frohnmeyer *et al.*, 1997) indicate that increased $[Ca^{2+}]_{cyt}$ is a component of UV/blue light signal transduction pathways regulating gene expression. However, as this evidence is indirect, it was important to establish whether UV-A/blue light stimulated an increase in $[Ca^{2+}]_{cyt}$. It was also valuable to determine whether any observed UV-A/blue light induced increase in $[Ca^{2+}]_{cyt}$ was mediated by CRY1. Therefore, *Arabidopsis thaliana* (Landsberg ecotype) and *Arabidopsis hy4-2.23N* mutant plants were stably transformed (as described in 2.12) with a cauliflower mosaic virus 35S promoter apoaequorin gene construct in the binary plasmid, pMAQ2, donated by Dr. M.R. Knight (Oxford University, UK).

Seven wild-type and six *hy4* kanamycin resistance T_1 transformants were obtained and allowed to self-pollinate. T_2 wild-type line 3 and *hy4* line 1 displayed a 3:1 ratio of Kan^R as expected for a single locus of insertion. Homozygous progeny lines, L3:1 and L1:10 from wild-type and *hy4*, respectively, were chosen for further analysis.

6.3 Cold-shock response of wild-type and *hy4* transgenic plants

Cold-shock and mechanical-stimulation increase $[Ca^{2+}]_{cyt}$ in *Arabidopsis* (Knight *et al.*, 1991; Knight *et al.*, 1996). Therefore, expression of the aequorin photoprotein in transformants was assessed by measurement of cold- and touch-induced luminescence from 10 day old seedlings after overnight reconstitution with cp-coelenterazine. Ice-

cold water was injected onto the seedlings to give a combination of touch-and cold-shock. Luminescence was counted for 1 minute.

Figure 6.1A and B shows that wild-type (L3:1) and *hy4* (L1:10) plants displayed strong aequorin expression. Therefore, these lines were used in all further studies.

6.4 UV-A/blue light induced $[Ca^{2+}]_{cyt}$ increase in *Arabidopsis*

No increase in $[Ca^{2+}]_{cyt}$ as a response to UV-A/blue light has been reported, although previous studies on the effect of UV-A/blue light on $[Ca^{2+}]_{cyt}$ in transgenic *Arabidopsis* aequorin seedlings have been undertaken (Christie and Jenkins, 1996; Lewis *et al.*, 1997). Christie and Jenkins (1996) used single seedlings in their preliminary measurements whereas Lewis *et al.* (1997) used multiple seedlings, but gave a low fluence rate of blue light (100 µmol m^{-2} s^{-1}). Both studies proposed that a UV-A/blue light induced $[Ca^{2+}]_{cyt}$ increase may occur in a subset of cells, and this increase may therefore be difficult to detect in whole seedlings.

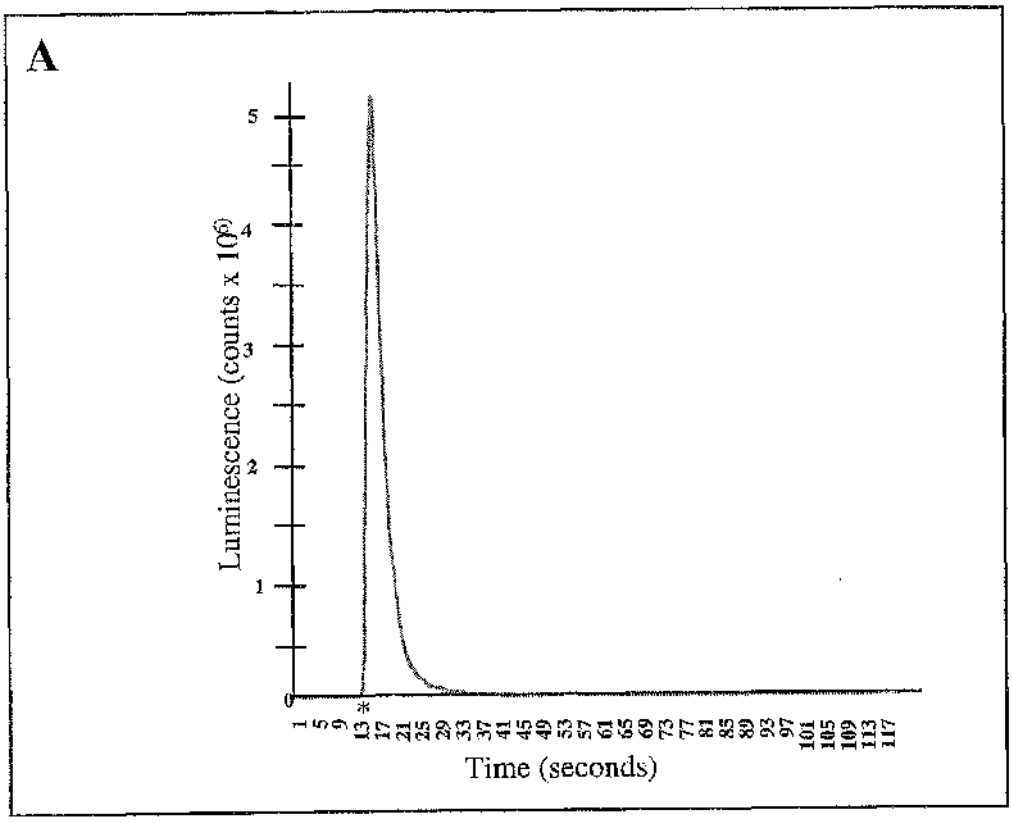
Therefore, in these experiments the effect of high fluence rate (650 µmol m^{-2} s^{-1}) UV-A/blue light (Figure 2.2E) on multiple seedlings was examined. Additionally, in order to increase the sensitivity of reconstituted aequorin, the synthetic analogue cp-coelenterazine, which is more sensitive to $[Ca^{2+}]_{cyt}$, was used (Shimomura *et al.*, 1993). *Arabidopsis* seedlings were illuminated for 10 secs with UV-A/blue light. Luminescence was converted into nM $[Ca^{2+}]_{cyt}$ as described in Section 2.12.3

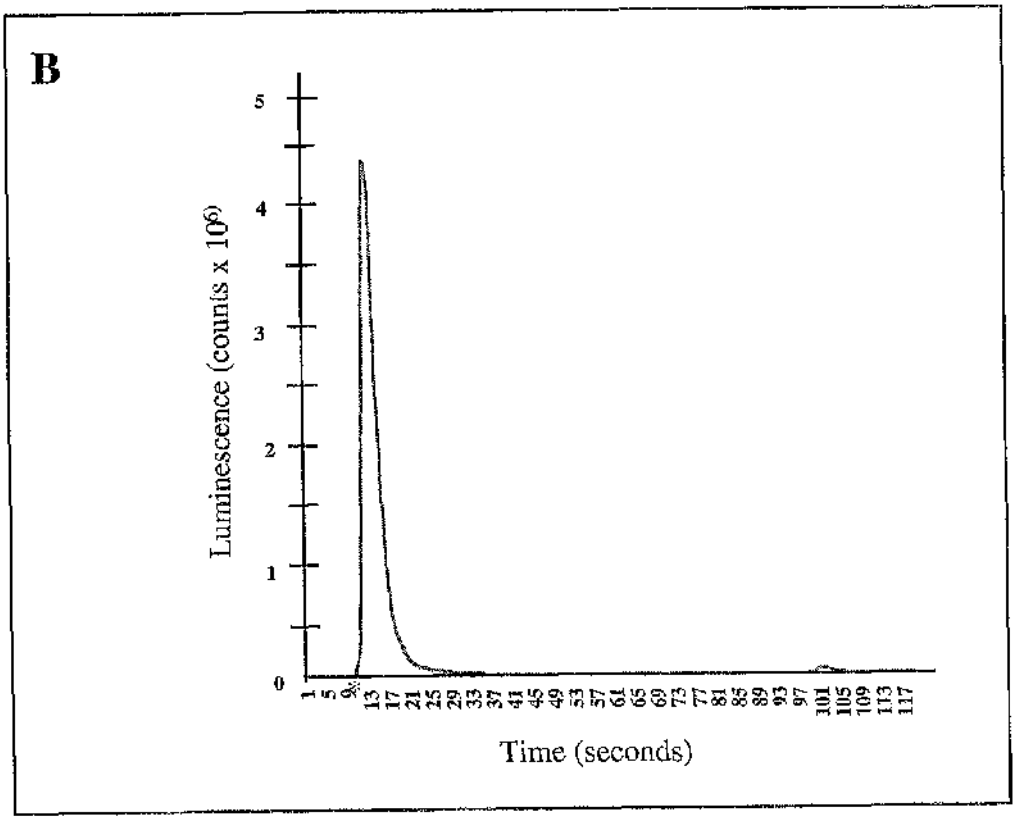
Figure 6.2 shows a representative response to UV-A/blue light. The data collected during the first 22 secs of the experiments, along with the final calculated $[Ca^{2+}]_{cyt}$ is shown in Table 6.1. $[Ca^{2+}]_{cyt}$ started to increase immediately after illumination, with no lag period. A single peak of $[Ca^{2+}]_{cyt}$ was observed. $[Ca^{2+}]_{cyt}$

Figure 6.1 Cold shock response in transformants

(A) 10-day old wild-type *Arabidopsis* L3:1 seedlings grown in 25 μmol m^{-2} s^{-1} white light were incubated in cp-coelenterazine overnight. Seedlings were transferred to the luminometer in total darkness and basal levels measured for 3 mins. Ice-cold water was injected onto the plants, at the time indicated by (*), and luminescence recorded.

(B) 10-day old *hy4* L1:10 seedlings grown in 25 μmol m^{-2} s^{-1} white light were incubated in cp-coelenterazine overnight. Seedlings were transferred to the luminometer in total darkness and basal levels measured for 3 mins. Ice-cold water was injected onto the plants, at the time indicated by (*), and luminescence recorded.


A
Luminescence (counts x 10⁶)
0
1
2
3
4
5
1
5
9
13
17
21
25
29
33
37
41
45
49
53
57
61
65
69
73
77
81
85
89
93
97
101
105
109
113
117
Time (seconds)


B
Luminescence (counts x 10⁶)
0
1
2
3
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1
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33
37
41
45
49
53
57
61
65
69
73
77
81
85
89
93
97
101
105
109
113
117
Time (seconds)

Figure 6.2 UV-A/blue light induced $[Ca^{2+}]_{cyt}$ response in wild-type *Arabidopsis*

10 day old wild-type *Arabidopsis* L3:1 seedlings grown in 25 μmol m^{-2} s^{-1} white light were incubated in cp-colenterazine overnight in darkness. Seedlings were transferred to the luminometer in total darkness and basal levels measured for 3 mins, as indicated in the figure by basal $[Ca^{2+}]_{cyt}$. Seedlings were illuminated with 650 μmol m^{-2} s^{-1} UV-A/blue light for 10 secs and luminescence recorded. Subsequently, ice-cold water was injected onto the plants and luminescence recorded for 1 min. nM $[Ca^{2+}]_{cyt}$ was calculated from luminescence as described in 2.12.4. The figure shows the calculated change in $[Ca^{2+}]_{cyt}$ from the point when illumination with UV-A/blue light ceased, and the basal $[Ca^{2+}]_{cyt}$ prior to illumination.

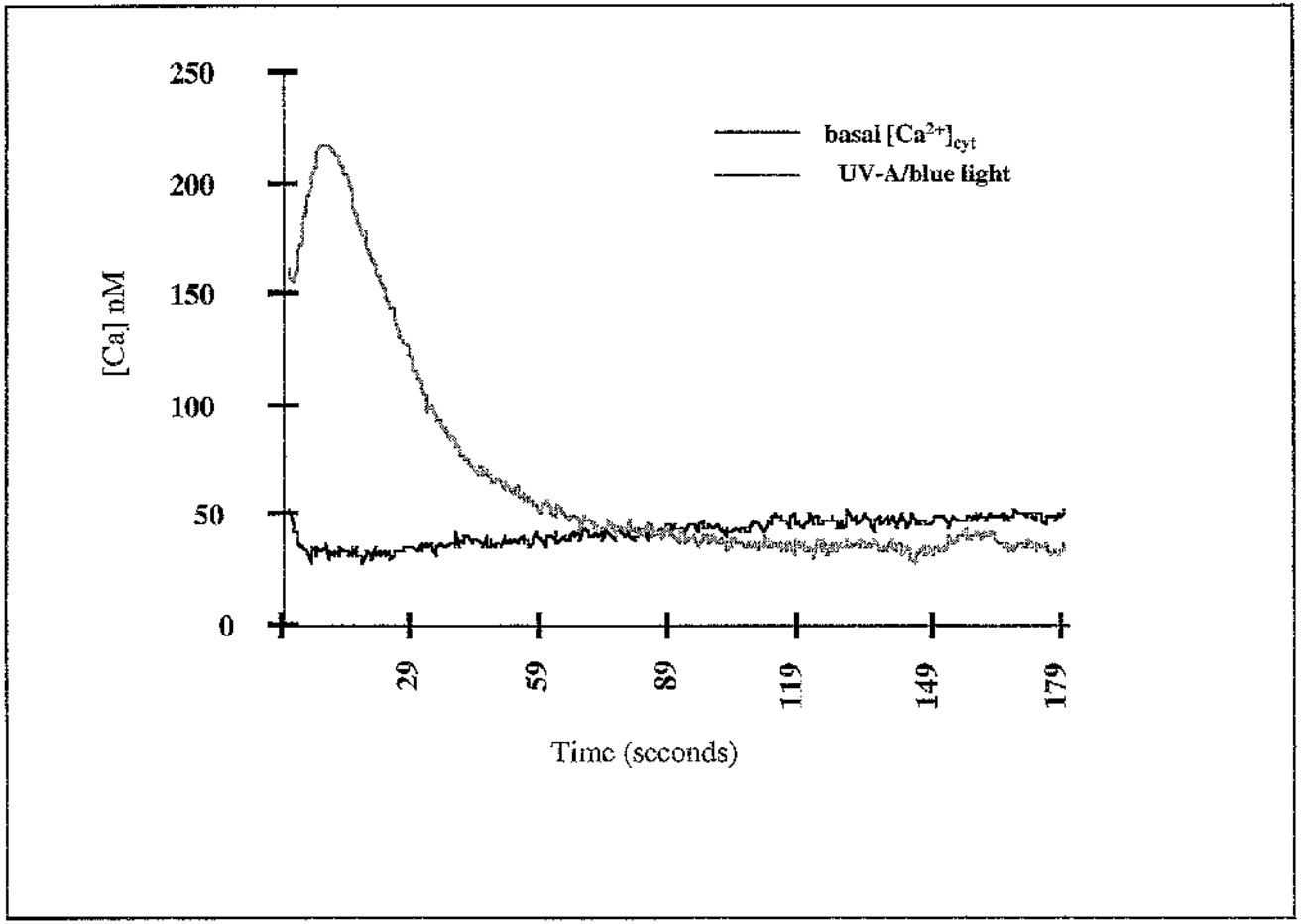

250
200
150
100
50
0
[Ca] nM
basal [Ca2+]cyt
UV-A/blue light
29
59
89
119
149
179
Time (seconds)

Table 6.1 Data from Figure 6.2

The table shown opposite is the data collected during the first 22 secs of the experiment shown in Figure 6.2. The treatment referred to in the table is 10 secs of UV-A/blue light (650 μmol m^{-2} s^{-1}). The cold-shock response was administered at 7 secs by injection of ice-cold water onto the seedlings as described in Section 2.12.2. The nm $[Ca^{2+}]_{cyt}$ was calculated from all of the data from the entire 3 min experiment, as described in 2.12.4, and the $[Ca^{2+}]_{cyt}$ pre- and post-treatment with UV-A/blue light is shown in the final 2 columns.

Time (secs)	pre-treatment counts	post-treatment counts	cold-shock treatment counts	pre-treatment $[Ca^{2+}]_{cyt}$ nM	post-treatment $[Ca^{2+}]_{cyt}$ nM
0	478	4351	292	52	161
0.5	479	4124	266	52	157
1	400	4055	260	46	156
1.5	343	4097	254	41	157
2	351	4333	279	42	161
2.5	286	4883	274	36	170
3	271	5155	270	35	174
3.5	277	5713	284	35	182
4	248	6163	277	33	188
4.5	263	6441	254	34	192
5	206	6881	255	28	198
5.5	277	7219	246	35	202
6	248	7691	254	33	208
6.5	245	8038	231	32	212
7	271	8147	48438	35	213
7.5	251	8407	950515	33	216
8	261	8484	2032409	34	217
8.5	265	8480	2152347	34	217
9	292	8488	1769424	37	217
9.5	225	8382	1411617	30	216
10	260	8446	1103993	34	217
10.5	244	8182	859318	32	214
11	263	8102	668620	34	213
11.5	236	7942	524215	31	211
12	264	7757	416961	34	209
12.5	251	7506	334902	33	206
13	248	7407	272665	33	205
13.5	254	7219	223936	33	203
14	241	7044	188063	32	201
14.5	255	6638	159182	33	195
15	271	6271	136458	35	191
15.5	236	6176	118121	31	189
16	263	5896	102132	34	186
16.5	234	5664	89449	31	182
17	236	5399	78336	31	178
17.5	201	5271	68816	27	177
18	230	5353	60635	31	178
18.5	247	4939	53297	32	172
19	238	4627	47145	31	167
19.5	229	4622	41269	31	167
20	275	4417	37496	35	163
20.5	239	4206	33291	32	160
21	261	4071	29926	34	157
21.5	242	4009	27035	32	156
22	246	3792	24257	32	152
22.5	258	3598	21854	34	149

peaked between 10-20 secs after illumination, before almost immediately (5-10 secs) beginning to decline, reaching original $[Ca^{2+}]_{cyt}$ resting levels at around 70-80 secs after UV-A/blue illumination. $[Ca^{2+}]_{cyt}$ then decreased slightly below the resting level. Although the UV-A/blue light induced increase in $[Ca^{2+}]_{cyt}$ was observed in all seedlings analyzed, some plant-to-plant variation in the magnitude of $[Ca^{2+}]_{cyt}$ peak was observed. Also, a decrease in $[Ca^{2+}]_{cyt}$ below the original level after the response was not always observed. Red light had no effect on $[Ca^{2+}]_{cyt}$ (data not shown).

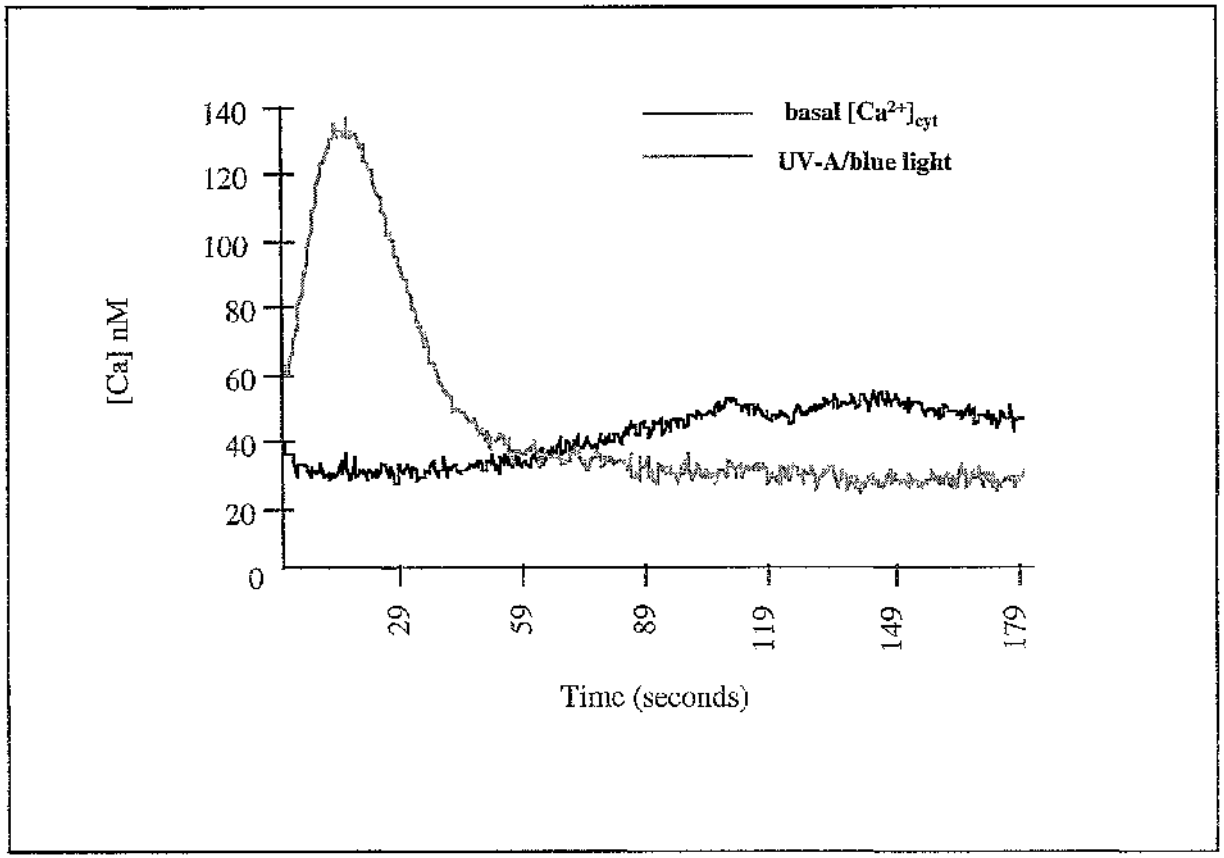
6.5 UV-A/blue light induced $[Ca^{2+}]_{cyt}$ increase in *hy4*

To determine whether the UV-A/blue light induced response is mediated by CRY1, the transformed *hy4* plants (L1:10) were illuminated with UV-A/blue light for 10 secs.

Figure 6.3 shows that the response is also present in *hy4*. As in wild-type, there was also a single peak with no lag period and the peak in $[Ca^{2+}]_{cyt}$ occurred 10-20 secs after illumination. Again, $[Ca^{2+}]_{cyt}$ appeared to decrease below the original concentration in most, but not all, seedlings. There was also variation between experiments in the magnitude of the UV-A/blue induced peak in $[Ca^{2+}]_{cyt}$, and Figure 6.3 is representative of the response. Although we did observe a slightly smaller increase in UV-A/blue light induced $[Ca^{2+}]_{cyt}$ in these preliminary studies, subsequent studies have confirmed that there is no significant difference in the UV-A/blue light induced increase in $[Ca^{2+}]_{cyt}$ between wild-type and *hy4 Arabidopsis* seedlings (Dr. G. Baum, personal communication). This result indicates that CRY1 does not mediate the UV-A/blue light response.

Figure 6.3 UV-A/blue light induced $[Ca^{2+}]_{cyt}$ response in *hy4*

10 day old *Arabidopsis hy4* L1:10 seedlings grown in 25 μmol m^{-2} s^{-1} white light were incubated in cp-colenterazine overnight in darkness. Seedlings were transferred to the luminometer in total darkness and basal levels measured for 3 mins, as indicated in the figure by basal $[Ca^{2+}]_{cyt}$. Seedlings were illuminated with 650 μmol m^{-2} s^{-1} UV-A/blue light for 10 secs and luminescence recorded. Subsequently, ice-cold water was injected onto the plants and luminescence recorded for 1 min. nM $[Ca^{2+}]_{cyt}$ was calculated from luminescence as described in 2.12.4. The figure shows the calculated change in $[Ca^{2+}]_{cyt}$ from the point when illumination with UV-A/blue light ceased, and the basal $[Ca^{2+}]_{cyt}$ prior to illumination.


basal [Ca2+]cyt
UV-A/blue light
[Ca] nM
140
120
100
80
60
40
20
0
29
59
89
119
149
179
Time (seconds)

6.6 EGTA alters the UV-A/blue light induced $[Ca^{2+}]_{cyt}$ increase

It was important to determine the pool of Ca^{2+} responsible for the UV-A/blue response. EGTA is an impermeable Ca^{2+} chelator, and has been used to study exogenous Ca^{2+} in aequorin measured $[Ca^{2+}]_{cyt}$ increases in response to environmental stimuli (Knight *et al.*, 1996; Haley *et al.*, 1995; Sedbrook *et al.*, 1996; Price *et al.*, 1994). Therefore the UV-A/blue light induced $[Ca^{2+}]_{cyt}$ increase was examined in 12 day old *Arabidopsis* seedlings pre-treated for one hour with EGTA.

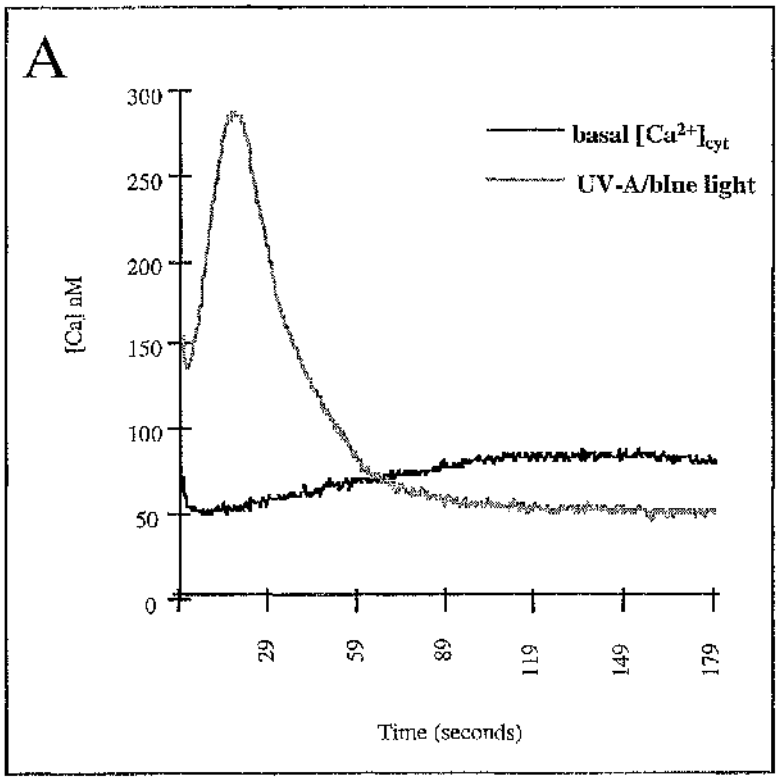
It was regularly observed that the UV-A/blue light induced increase in $[Ca^{2+}]_{cyt}$ was greater in older seedlings. Therefore a control response with 12 day old seedlings is shown in Figure 6.4A. The results shown in Figure 6.4B indicated that EGTA partially inhibited the magnitude and kinetics of the UV-A/blue light response, compared to the control (Figure 6.4A). The UV-A/blue induced peak in $[Ca^{2+}]_{cyt}$ occurred at 30-35 secs in the presence of EGTA. The peak in $[Ca^{2+}]_{cyt}$ was prolonged over 15-20 secs before decreasing back to the original $[Ca^{2+}]_{cyt}$. The magnitude of the increase in $[Ca^{2+}]_{cyt}$ is also much lower than in the untreated control.

A partial inhibitory effect of EGTA was also reported for cold-shock (Knight *et al.*, 1996) and anoxia (Sedbrook *et al.*, 1996) regulated $[Ca^{2+}]_{cyt}$ increases. EGTA would be expected to chelate Ca^{2+} in the cell wall, but may not completely inhibit Ca^{2+} channel activity.

Figure 6.4 Effect of EGTA on the UV-A/blue light induced $[Ca^{2+}]_{cyt}$ response

(A) 12 day old wild-type *Arabidopsis* L3:1 seedlings grown in 25 μmol m^{-2} s^{-1} white light were incubated in cp-colenterazine overnight in darkness. Seedlings were transferred to the luminometer in total darkness and basal levels measured for 3 mins, as indicated in the figure by basal $[Ca^{2+}]_{cyt}$. Seedlings were illuminated with 650 μmol m^{-2} s^{-1} UV-A/blue light for 10 secs and luminescence recorded. Subsequently, ice-cold water was injected onto the plants and luminescence recorded for 1 min. nM $[Ca^{2+}]_{cyt}$ was calculated from luminescence as described in 2.12.4. The figure shows the calculated change in $[Ca^{2+}]_{cyt}$ from the point when illumination with UV-A/blue light ceased, and the basal $[Ca^{2+}]_{cyt}$ prior to illumination.

(B) 12 day old *Arabidopsis* seedlings grown in 25 μmol m^{-2} s^{-1} white light were incubated in 10 mM EGTA for one hour, followed by cp-colenterazine overnight in darkness. Luminescence was measured as in (A).


A
300
250
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[Ca] nM
basal [Ca2+]cyt
UV-A/blue light
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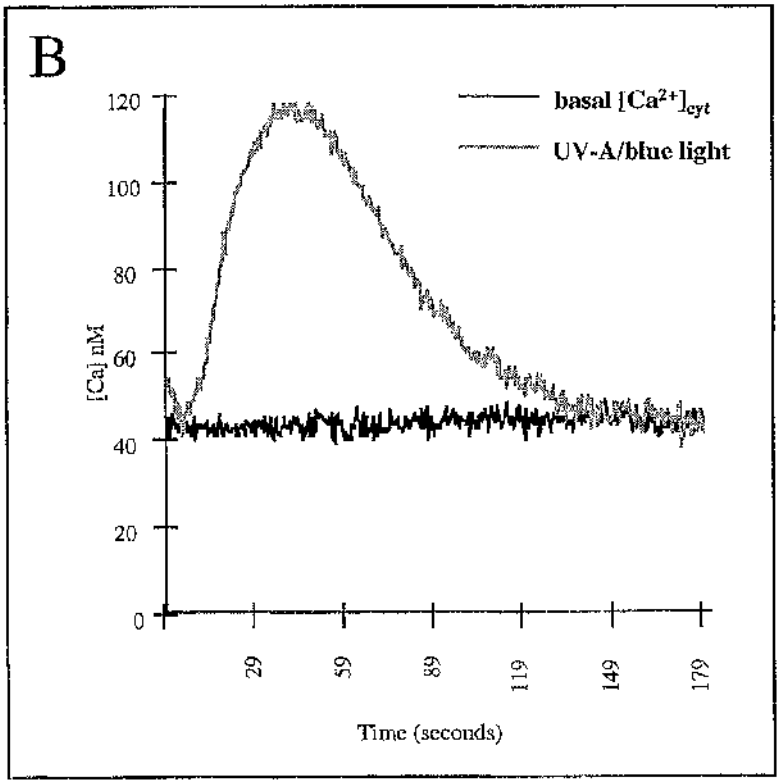
Time (seconds)


B
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100
80
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20
0
[Ca] nM
basal [Ca2+]cyt
UV-A/blue light
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Time (seconds)

6.7 Lanthanum and ruthenium red inhibit the UV-A/blue light induced $[Ca^{2+}]_{cyt}$ increase

The above result indicated that extracellular Ca^{2+} mediates the UV-A/blue light regulated $[Ca^{2+}]_{cyt}$ increase. To further investigate the response, the effect of Ca^{2+} channel inhibitors was examined. Lanthanum is an impermeable inhibitor of Ca^{2+} channels, and has been used with transgenic aequorin plants to study plasma membrane Ca^{2+} channels in responses to various stimuli (Knight *et al.*, 1992; Knight *et al.*, 1996; Haley *et al.*, 1995; Price *et al.*, 1994; Sedbrook *et al.*, 1996). To determine whether plasma membrane Ca^{2+} channels are involved in the UV-A/blue light response, we pre-treated seedlings for one hour with lanthanum prior to illumination with UV-A/blue light.

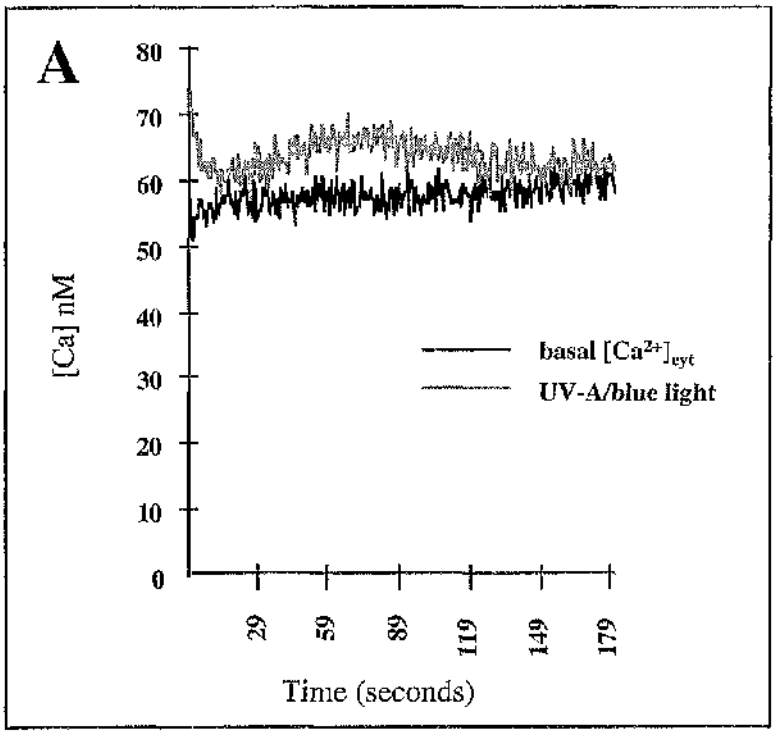
Figure 6.5A shows that lanthanum completely inhibited the response. Together with the effect of EGTA, this result suggests that the UV-A/blue light induced increase in $[Ca^{2+}]_{cyt}$ is mediated by an influx of extracellular Ca^{2+} through activation of plasma membrane Ca^{2+} channels.

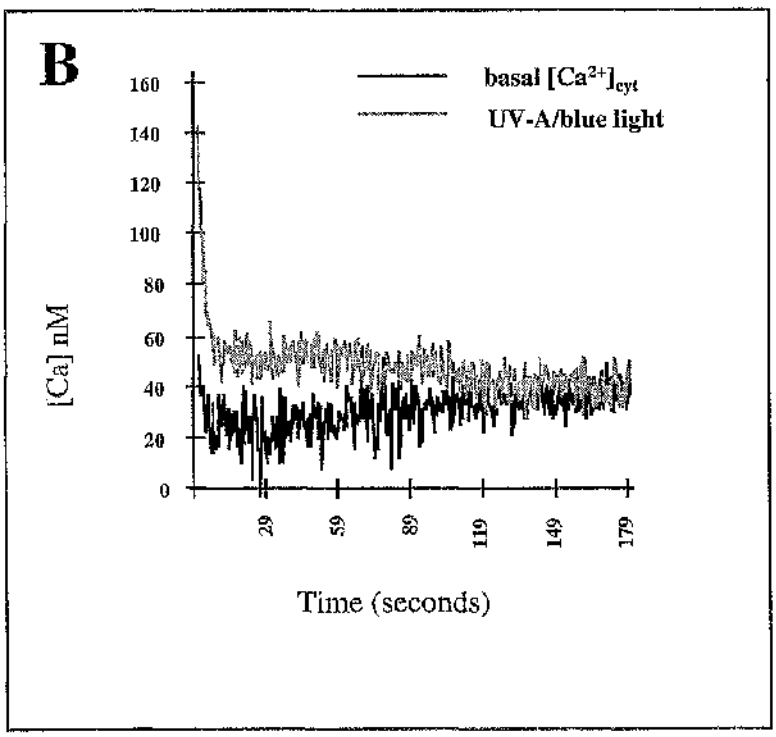
The effect of the Ca^{2+} channel blocker, ruthenium red, on the UV-A/blue light induced $[Ca^{2+}]_{cyt}$ increase was also examined. Ruthenium red has been used to study the contribution of intracellular stores of Ca^{2+} to increases in $[Ca^{2+}]_{cyt}$ measured using aequorin in response to various stimuli (Knight *et al.*, 1992; Haley *et al.*, 1995; Price *et al.*, 1994; Sedbrook *et al.*, 1996). Figure 6.5B shows that ruthenium red had the same effect as lanthanum. The most likely interpretation is that ruthenium red can inhibit plasma membrane Ca^{2+} channels, as well as intracellular channels, as indicated by Marshall *et al.* (1994). Therefore, caution should be exercised in concluding that intracellular Ca^{2+} stores are involved in a response based solely on an inhibitory effect of ruthenium red.

Figure 6.5 Effect of lanthanum and ruthenium red on the UV-A/blue light induced $[Ca^{2+}]_{cyt}$ response

(A) 12 day old wild-type *Arabidopsis* L3:1 seedlings grown in 25 μmol m^{-2} s^{-1} white light were incubated in 3 mM lanthanum for one hour, followed by cp-colenterazine overnight in darkness. Seedlings were illuminated with 650 μmol m^{-2} s^{-1} UV-A/blue light for 10 secs and luminescence recorded. Subsequently, ice-cold water was injected onto the plants and luminescence recorded for 1 min. nM $[Ca^{2+}]_{cyt}$ was calculated from luminescence as described in 2.12.4. The figure shows the calculated change in $[Ca^{2+}]_{cyt}$ from the point when illumination with UV-A/blue light ceased, and the basal $[Ca^{2+}]_{cyt}$ prior to illumination.

(B) 12 day old *Arabidopsis* seedlings grown in 25 μmol m^{-2} s^{-1} white light were incubated in 50 μM ruthenium red for one hour, followed by cp-colenterazine overnight in darkness. Luminescence was measured as in (A).


A
80
70
60
50
40
30
20
10
0
[Ca] nM
basal [Ca2+]cyt
UV-A/blue light
29
59
89
119
149
179
Time (seconds)


B
160
140
120
100
80
60
40
20
0
[Ca] nM
basal [Ca2+]cyt
UV-A/blue light
29
59
89
119
149
179
Time (seconds)

6.8 Discussion

In this chapter transgenic *Arabidopsis* wild-type and *hy4* seedlings expressing cytosolic aequorin were generated. UV-A/blue light rapidly induced an increase in cytosolic Ca^{2+} concentration. This response was characterised and we were able to demonstrate that the Ca^{2+} increase was independent of the UV-A/blue light photoreceptor, CRY1, and requires external Ca^{2+}.

6.8.1 The UV-A/blue light induced $[Ca^{2+}]_{cyt}$ increase is independent of CRY1

The results in this study and previous work (Christe and Jenkins, 1996; Frohnmeyer *et al.*, 1997) indicate a role for $[Ca^{2+}]_{cyt}$ in UV-A/blue and UV-B light signalling pathways regulating gene expression. Additionally, phytochrome regulation of $[Ca^{2+}]_{cyt}$ has been implicated in the control of gene expression (Neuhaus *et al.*, 1993). In *Arabidopsis* cells, pharmacological investigations suggest that UV/blue light regulated *CHS* expression requires a Ca^{2+} influx via a nifedipine-sensitive, lanthanum-insensitive Ca^{2+} channel (Christie and Jenkins, 1996) and Ca^{2+} efflux via activation of a Ca^{2+}-ATPase (this study). However, it is important to study UV/blue light regulated changes in $[Ca^{2+}]_{cyt}$ directly in order to further investigate the location and gating properties of the UV/blue regulated Ca^{2+} influx and efflux components, as well as the kinetics and magnitude of the proposed $[Ca^{2+}]_{cyt}$ increase regulating *CHS* in *Arabidopsis*.

Although cytosolic Ca^{2+} fluxes are generally measured using Ca^{2+} indicator dyes in mammalian cells, there are problems with this approach in plant cells. The most significant problems are dye entry into plant cells and dye compartmentation

inside the cell (Read *et al.*, 1993). However, a novel method has been developed to study $[Ca^{2+}]_{cyt}$ in plant cells which avoids these problems. Aequorin is a Ca^{2+} binding protein from *Aequoria victoria* which emits photons of blue light at a rate dependent on $[Ca^{2+}]_{cyt}$. The aequorin photoprotein has two components: a polypeptide apoaequorin, and a luminophore, coelenterazine. Transgenic tobacco plants expressing cytosolic apoaequorin have been generated which, after in vivo reconstitution of functional aequorin with coelenterazine, were used to show a rapid increase in $[Ca^{2+}]_{cyt}$ as a response to cold-, elicitor- and mechanical stimulation (Knight *et al.*, 1991). Recently, aequorin technology has been used successfully to study $[Ca^{2+}]_{cyt}$ increases in tobacco and *Arabidopsis* in response to a variety of external stimuli (Knight *et al.*, 1996; Johnson *et al.*, 1995; Sedbrook *et al.*, 1996; Knight *et al.*, 1992; Knight *et al.*, 1995; Takahashi *et al.*, 1997; Price *et al.*, 1994). In addition to avoiding problems with dyes, aequorin technology has other advantages. A number of synthetic coelenterazine analogues have been produced, which enables production of aequorins with different sensitivities (Shimomura *et al.*, 1993). Also, transgenic plants have been produced with aequorin targetted to specific intracellular compartments (Knight *et al.*, 1996).

Although transgenic *Arabidopsis* cv. Columbia plants have already been produced, we were interested in studying UV-A/blue regulated $[Ca^{2+}]_{cyt}$ increases in the *Arabidopsis hy4* mutant, which is in the Landsberg ecotype. Therefore, transgenic *Arabidopsis* Landsberg and *hy4* were produced in order to compare UV-A/blue responses in the same genetic background. Transgenic *Arabidopsis* and *hy4* lines which expressed strong aequorin activity were obtained (Figure 6.1) and used throughout this study.

Recently, several attempts have failed to find a UV-A/blue light increase in $[Ca^{2+}]_{cyt}$ in *Arabidopsis* seedlings expressing cytosolic aequorin (Christie and Jenkins, 1996; Lewis *et al.*, 1997). In this study we used multiple seedlings to increase the detectable light emission, a high fluence rate of UV-A/blue light, and the synthetic

analogue, cp-coelenterazine (Shimomura *et al.*, 1993), for *in vivo* reconstitution of aequorin, producing aequorin with increased sensitivity to $[Ca^{2+}]_{cyt}$.

A rapid increase in $[Ca^{2+}]_{cyt}$, observed as a single peak, was detected in transgenic *Arabidopsis* seedlings illuminated with high fluence UV-A/blue light (Figure 6.2). It was observed that the level of $[Ca^{2+}]_{cyt}$ fell below the resting concentration after UV-A/blue illumination although this result varied between plants, and more studies need to be carried out to quantify the effect. Red light had no effect on $[Ca^{2+}]_{cyt}$ (data not shown). The response is present in the *hy4* mutant, indicating that the response is independent of CRY1 (Figure 6.3). Additionally, the response is altered by EGTA (Figure 6.4) and inhibited by the impermeable Ca^{2+} channel blocker, lanthanum (Figure 6.5), indicating that external Ca^{2+} mediates the response. The UV-A/blue light induction of *CHS* in *Arabidopsis* cells is not mediated by CRY1 and is inhibited by concentrations of lanthanum that inhibit the cantharidin induction of *PAL* (Christie and Jenkins, 1996). Therefore these results indicate that the rapid UV-A/blue regulated increase in $[Ca^{2+}]_{cyt}$ does not mediate *CHS* expression in *Arabidopsis*. The increase in $[Ca^{2+}]_{cyt}$ is presumably related to some other UV-A/blue light response. This could be a gene expression response or, for example, an extension growth response. The origin of the aequorin luminescence within the seedlings is not yet known.

Blue light induces a rapid plasma membrane depolarization of etiolated *Arabidopsis* hypocotyl cells, preceeding growth inhibition (Cho and Spalding, 1996). This response has been proposed to be mediated by an anion channel (Cho and Spalding, 1996), and may be regulated by CRY1 as *hy4* seedlings are impaired in blue light regulated growth suppression (Ahmad and Cashmore, 1993). Lewis *et al.* (1997) reported that there was no increase in $[Ca^{2+}]_{cyt}$ by blue light in etiolated *Arabidopsis* seedlings, and therefore proposed that anion channel activity is not induced by $[Ca^{2+}]_{cyt}$. The response reported in this study is most likely not involved in

blue light-induced membrane depolarization because the response is CRY1 independent, and interestingly is not present in etiolated seedlings (data not shown; G.Baum, personal communication). Unfortunately, due to time limitations, we were unable to investigate the role of the UV-A/blue light response in the regulation of other genes in *Arabidopsis*. However, it will be impotant to identify the physiological role for the UV-A/blue response in the future.

Therefore, these studies need to be continued in order to identify the UV-A/blue light regulated Ca^{2+} pool involved in *CHS* expression in *Arabidopsis*. Additionally, we did not examine the effect of UV-B light on $[Ca^{2+}]_{cyt}$, and this will be a priority in future studies. However, this study has demonstrated that aequorin technology, together with a genetic approach, can be used to in analyse UV-A/blue light regulated $[Ca^{2+}]_{cyt}$ in *Arabidopsis* seedlings.

CHAPTER 7: FINAL DISCUSSION

7.1 Introduction

Pharmacological analysis of UV/blue light signal transduction pathways was carried out on *P.vulgaris* protoplasts and *Arabidopsis* cells and plants. Additionally, preliminary characterisation of a UV-A/blue light regulated $[Ca^{2+}]_{cyt}$ increase in *Arabidopsis* plants was undertaken. The aim of this chapter is to discuss and draw together the main conclusions on UV-A/blue and UV-B signal transduction components and to summarise the prospects for future work.

7.2 Components involved in UV-A/blue light regulation of *rbcS* in *P.vulgaris* protoplasts

In this study protoplasts isolated from dark-adapted light-grown mature leaves of *P.vulgaris* were used to investigate UV-A/blue light signal transduction. The protoplasts show the same response to different light qualities as intact leaves (Sawbridge *et al.*, 1994). *rbcS* transcripts are induced by UV-A/blue light rather than red light (Figure 3.1). The UV-A/blue light signalling pathway regulating *rbcS* appears to require cellular Ca^{2+}, as the Ca^{2+} channel blocker, nifedipine, inhibits transcript accumulation (Figure 3.3). This suggests that a voltage-gated Ca^{2+} channel is involved. It is important to identify the Ca^{2+} channel involved and determine its location and its gating properties in the cell. The Ca^{2+}-binding protein, calmodulin, does not seem to be involved (Figure 3.4). Additionally, protein phosphorylation (Figure 3.5) and dephosphorylation are required (Figure 3.6). Evidence was also obtained that *rbcS* induction by UV-A/blue light requires protein synthesis (Figure 3.7).

Pharmacological experiments, such as those described in this study, require the appropriate controls to show: (i) that the compound is entering the cell; (ii) the compound is having an effect on the appropriate components, and (iii) the effect described is not due to a general inhibitory effect on normal cell processes. Unfortunately, we did not have an appropriate control for the experiments described in Chapter 3. The concentrations of inhibitors used in this study were not toxic to the protoplasts. However W7, genistein, verapamil and ruthenium red, which did not have any effect on UV-A/blue induced *rbcS* expression were toxic to the protoplasts at concentrations above those in this study (data not shown). This suggests that the compounds are entering the cells and inhibiting cellular components. Cycloheximide, nifedipine, staurosporine and cantharidin inhibited UV-A/blue light induced *rbcS* expression, but we were unable to show that this was not due to a detrimental effect on normal processes. It is promising, however, to note that these compounds also inhibited the UV-A/blue light induction of *CHS* in *Arabidopsis* cells. These compounds were not having a general inhibitory effect on *Arabidopsis* cells as they did not inhibit control responses (Christie and Jenkins, 1996).

The results indicate that the UV-A/blue light signalling pathways regulating *rbcS* in *P.vulgaris* protoplasts and *CHS* in *Arabidopsis* cells are similar, suggesting that UV-A/blue light signal transduction pathways may be conserved between different species. The photoreceptor regulating *rbcS* in *P.vulgaris* has not been identified, but may be homologous to the *Arabidopsis* CRY1 photoreceptor.

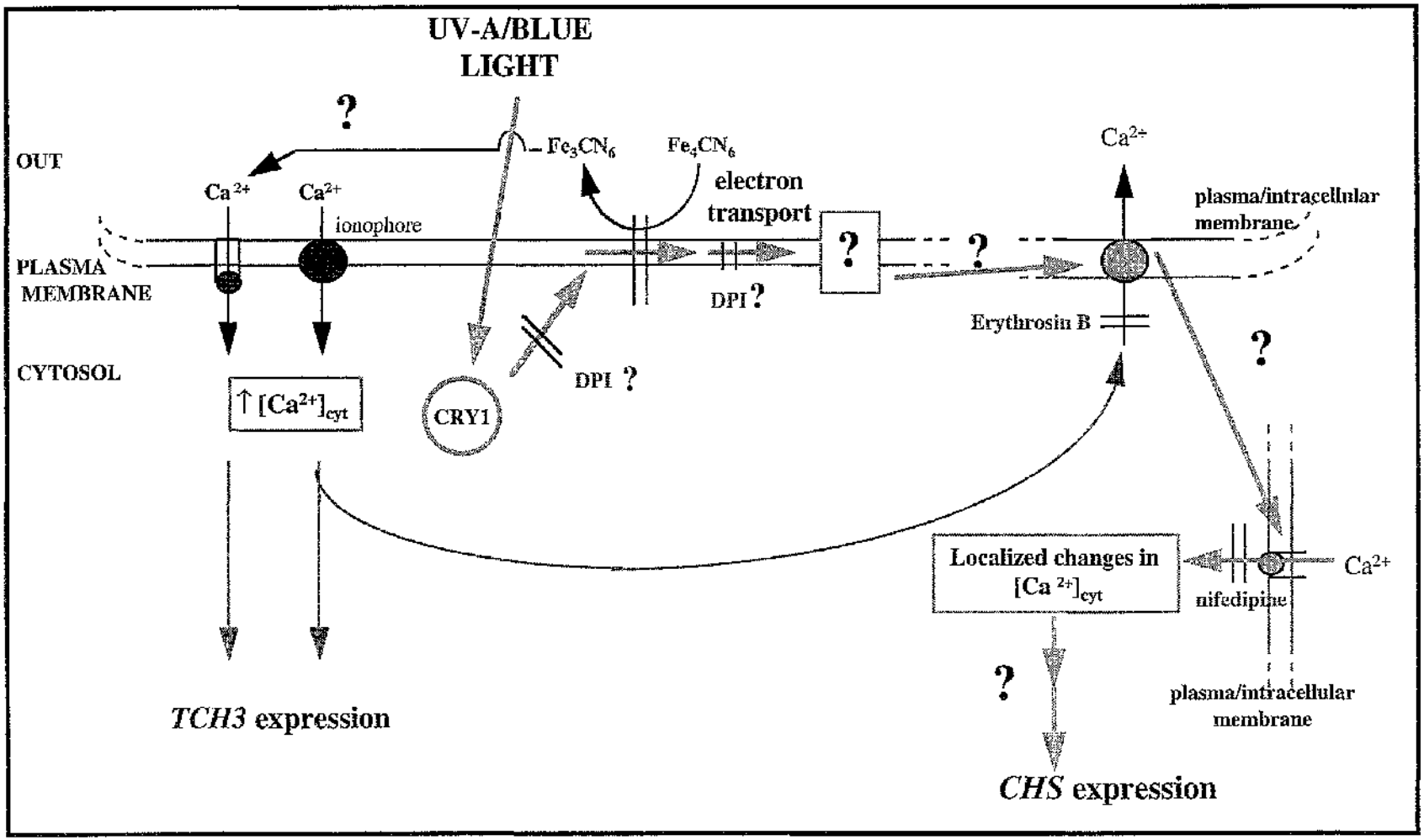
7.3 UV/blue light regulated redox processes regulating *CHS*

The CRY1 photoreceptor is a flavin-binding protein that has been proposed to initiate electron transfer as an early step in signal transduction (Lin *et al.*, 1995a; Ahmad and Cashmore, 1996). In this study it was found that the external electron acceptor, FeCN, inhibits UV-A/blue and UV-B light regulated *CHS* and *PAL* expression (Figure 4.2). This suggests that plasma membrane electron transport is initiated by CRY1 and the putative UV-B photoreceptor. FeCN had no effect on the cantharidin induction of *PAL* transcripts (Figure 4.3) and induced transcripts of *TCH3* in non-inductive white light (Figure 4.4), indicating that FeCN was not having a general effect on cell processes. *TCH3* encodes a novel calmodulin-like protein (Sistrunk *et al.*, 1994) which has been proposed to be induced by a variety of environmental signals by a stimulus-induced increase in cytosolic Ca^{2+} (Braam *et al.*, 1997). This suggests that FeCN, which is known to induce H^+ and K^+ efflux and plasma membrane depolarization, also stimulates Ca^{2+} influx. It was demonstrated that an ionophore-mediated increase in cytosolic Ca^{2+}, however, did not inhibit the UV/blue light induction of *CHS* (Figure 4.10). It is therefore proposed that FeCN is directly inhibiting UV-A/blue and UV-B induced *CHS* expression by interfering with plasma membrane electron transport.

From the above results, therefore, it is proposed that activated CRY1 and the putative UV-B photoreceptor indirectly or directly reduce a plasma membrane redox protein, as depicted for CRY1 in Figure 7.1. The plasma membrane contains oxidoreductases, which when activated produce reactive oxygen by electron transfer to molecular oxygen. The lipophilic flavoprotein inhibitor, DPI, inhibited UV-A/blue and UV-B light induced gene expression (Figure 4.7). This result suggested that a plasma membrane superoxide-generating protein could be involved in UV/blue light regulated gene expression. Oxidative stress has been demonstrated to regulate gene expression in plant cells (Levine *et al.*, 1994), most likely by altering the cellular redox status which can regulate transcription factor

Figure 7.1 Model of UV-A/blue light signal transduction pathway

The model illustrates the UV-A/blue light signalling pathway regulating *CHS* in *Arabidopsis* cells proposed in this study. In addition, the model shows the proposed pathways for the ionophore and FeCN induction of *TCH3* transcript accumulation in *Arabidopsis* cells.


UV-A/BLUE
LIGHT
?
OUT
Ca 2+
Ca2+
ionophore
PLASMA
MEMBRANE
CYTOSOL
↑ [Ca2+]cyt
CRY1
Fe3CN6
Fe4CN6
electron
transport
DPI ?
DPI ?
?
?
Ca2+
plasma/intracellular
membrane
Erythrosin B
?
Localized changes in
[Ca 2+]cyt
Ca2+
nifedipine
plasma/intracellular
membrane
?
TCH3 expression
CHS expression

activity. However, the results in Chapter 5 suggest that ROS generated by UV-A/blue and UV-B light are not involved, as compounds which would be predicted to alter cellular redox status had no effect. Additionally, it was determined that the cell culture environment may not be an appropriate system to study the regulation of genes involved in protection against oxidative stress, because of the levels of expression induced by mechanical stress. Possible controls for these experiments in *Arabidopsis* plants were identified. However, as ROS do not appear to be involved, we can only speculate on the site of action of DPI. As DPI inhibits reduced flavoproteins and is membrane permeable, DPI could presumably inhibit CRY1, as depicted in Figure 7.1. Alternatively it may inhibit a downstream flavoprotein in the putative electron transport chain. Although a UV-B photoreceptor has not been identified in *Arabidopsis*, flavins or pterins have been proposed for the chromophore (Jenkins *et al.*, 1997). It could be that DPI is having a similar effect on UV-B induced *CHS* , by inhibiting the putative photoreceptor.

An alternative role for plasma membrane redox activity in regulating gene expression could involve activation of a plasma membrane kinase. Blue light induced phosphorylation of a redox regulated plasma membrane protein, proposed to regulate phototropism, has been identified in maize coleoptiles (Hager *et al.*, 1993). Hager *et al.* (1993) proposed that reduction of the plasma membrane protein could induce a conformational change, possibly exposing phosphorylation sites on the target protein. It is possible that CRY1 and the proposed UV-B photoreceptor could transfer electrons to a protein in the plasma membrane which either becomes auto-phosphorylated or regulates the phosphorylation of another signal component, in a mechanism similar to that suggested by Hager *et al.* (1993). However, at present we do not know what the next step in the pathway is, as indicated in Figure 7.1.

7.4 UV/blue light regulated $[Ca^{2+}]_{cyt}$ responses regulating *CHS*

In this study, an important role for Ca^{2+}-ATPases in the UV-A/blue and UV-B light signalling pathways regulating *CHS* expression is proposed. Co-ordination of Ca^{2+} channels and Ca^{2+} efflux transporters at membrane locations in plant cells maintains the basal $[Ca^{2+}]_{cyt}$. An alteration is this Ca^{2+} concentration can be regulated by a variety of environmental signals (Bush, 1995). Although Ca^{2+} concentration is regulated by both Ca^{2+} influx channels and Ca^{2+} efflux transporters, there is less known about the regulation of Ca^{2+}-ATPases and their role in signal transduction pathways in plant cells than about the regulation of Ca^{2+} channels.

It was found that in cells treated with the Ca^{2+} ionophore, A23187, transfer to UV/blue light but not non-inductive white light reduced *TCH3* transcript accumulation (Figure 4.10). As described above, *TCH3* has been proposed to be induced by increased $[Ca^{2+}]_{cyt}$. A possible explanation for this unexpected result is that UV-A/blue light and UV-B light activate Ca^{2+} efflux transporters in *Arabidopsis* cells. In support of this model, the P-type Ca^{2+}-ATPase inhibitor, EB, inhibited UV-A/blue induced *CHS* expression (Figure 4.11).

At present, we do not have biochemical evidence for Ca^{2+}-ATPase activity regulated by UV/blue light. However, further pharmacological experiments were carried out to characterise the proposed UV/blue light regulated Ca^{2+}-ATPase activity, bearing in mind that these experiments represent indirect evidence. UV/blue regulated Ca^{2+}-ATPase activity was represented as the inhibition of ionophore-mediated *TCH3* transcript accumulation in *Arabidopsis* cells. It will be important to back-up these experiments with more direct biochemical evidence in the future.

It is proposed that UV-B light activates a Ca^{2+}-ATPase regulated by calmodulin, as W-7 inhibited the proposed Ca^{2+}-ATPase activity (Figure 4.12). Additionally, it is proposed that UV-A/blue and UV-B light regulated Ca^{2+}-ATPase

activities are upstream of calcium channel activity, as nifedipine did not inhibit the proposed Ca^{2+}-ATPase activity (Figure 4.13).

Therefore, the co-ordinated action of calcium pumps and channel activity is required for UV-A/blue and UV-B light regulated *CHS* expression in *Arabidopsis* cells. Recently, a Ca^{2+}-ATPase gene (*OsCa-atpase*) from rice, induced by gibberellin (GA), was isolated by differential display (Chen *et al.*, 1997). *OsCa-atpase* was demonstrated to regulate GA-signalling in aleurone cells and was proposed to regulate calcium channel activity by altering $[Ca^{2+}]_{cyt}$. Therefore, it is possible that UV/blue light activation of Ca^{2+}-ATPase could activate the nifedipine-sensitive calcium channel, as depicted in Figure 7.1. However this is only a model, and further understanding awaits molecular cloning of the proposed UV/blue regulated Ca^{2+}-ATPase(s) and biochemical characterisation of UV/blue light regulated Ca^{2+}-ATPase activity.

While providing information on possible signalling components concerned with the UV-A/blue and UV-B light induction of gene expression, experiments with pharmacological compounds have their limitations. It is important to employ cell physiological techniques to extend the information. In this study, aequorin technology was used to characterise a rapid UV-A/blue light regulated $[Ca^{2+}]_{cyt}$ increase. The response was initiated by 10 secs of high fluence rate UV-A/blue light (Figure 6.2). However, the UV-A/blue regulated $[Ca^{2+}]_{cyt}$ increase was also present in *hy4* seedlings (Figure 6.3), and was inhibited by lanthanum (Figure 6.5), suggesting that this response was independent of CRY1 and not involved in *CHS* expression. Future studies can be carried out to extend this work in order to identify a CRY1 mediated Ca^{2+} response, and also to characterise the proposed UV-B mediated $[Ca^{2+}]_{cyt}$ increase.

7.5 Common signalling components for UV-A/blue and UV-B light regulated *CHS* expression ?

UV-A/blue and UV-B light signalling pathways regulating *CHS* are distinct in their requirement for calmodulin in *Arabidopsis* cells (Christie and Jenkins, 1996). Additionally, in this study it was demonstrated that the kinetics of UV-A/blue and UV-B induced *CHS* expression are different (Figure 4.1), and the UV-B induction of *CHS* is insensitive to the P-type Ca^{2+}-ATPase inhibitor, EB (Figure 4.11). There are, however, a number of similarities between the two pathways. Christie and Jenkins (1996) demonstrated that the UV-A/blue and UV-B light signalling pathways regulating *CHS* both require Ca^{2+}, protein phosphorylation, and protein synthesis. In the present study it was demonstrated that the UV-B pathway is similar to the UV-A/blue pathway in that plasma membrane electron transport is required (Figure 4.2). Importantly, the UV-B pathway is inhibited by the flavoprotein inhibitor, DPI (Figure 4.7). A UV-B photoreceptor has not been isolated, although flavins or pterins have been proposed as a likely candidate for UV-B photoperception (Ensminger and Schafer, 1992; Khare and Guruprasad, 1993; Jenkins, 1997). Although the UV-B pathway is insensitive to EB, UV-B, like UV-A/blue light, inhibits the ionophore induction of *TCH3* (Figure 4.10). This suggests that a Ca^{2+}-ATPase is required, and it is proposed that a calmodulin-activated Ca^{2+}-ATPase is involved in the UV-B pathway.

UV-B has been proposed to regulate gene expression via the production of ROS (Green and Fluhr, 1995). In the present study, it is concluded that ROS are most likely not involved in the UV-B light regulation of *CHS*. The UV-B pathway requires signal components, such as kinase activity and $[Ca^{2+}]_{cyt}$ changes, characteristic of a complex signal transduction pathway. It will be important to identify the UV-B photoreceptor involved. It could be that the UV-A/blue and UV-B light signalling pathways share early components, but differ in downstream components.

7.6 Conclusions

The work presented in this study draws several conclusions about the UV/blue light signalling pathways regulating gene expression: (i) UV-A/blue light signal transduction pathways regulating *rbcS* in *P.vulgaris* and *CHS* in *Arabidopsis* appear similar in their requirement for Ca^{2+}, protein phosphorylation, and protein synthesis. (ii) UV-A/blue and UV-B light initiate electron transport at the plasma membrane which is required for *CHS* expression. (iii) A DPI-sensitive activated flavoprotein is a component of the UV-A/blue and UV-B signalling pathways. (iv) UV/blue light regulation of *CHS* does not require ROS. (v) UV-A/blue and UV-B light signal transduction results in an apparent decrease in the ionophore-mediated *TCH3* expression, by possibly decreasing ionophore-mediated $[Ca^{2+}]_{cyt}$. (vi) UV-A/blue light regulated *CHS* expression appears to require an EB-sensitive Ca^{2+}-ATPase. (vii) UV-B light appears to regulate a putative W7-sensitive Ca^{2+}-ATPase. (viii) A rapid UV-A/blue light induced $[Ca^{2+}]_{cyt}$ increase was identified but is not involved in CRY1 regulated *CHS* expression.

7.7 Future Work

This study has provided further information about UV-A/blue and UV-B light signalling components regulating gene expression. However, several areas are highlighted which are of particular interest and require further experiments. Plasma membrane electron transport appears to be an important component, probably reducing a plasma membrane signalling component. The production of ROS does not appear to be involved in regulating gene expression, suggesting that plasma membrane oxidoreductases are not involved. However, controls have been identified to test the effect of scavengers of ROS, and these experiments, along with direct measurements of the levels of ROS following UV/blue light, will

hopefully provide more definitive information. Phosphorylation of a plasma membrane protein has been suggested as an alternative mechanism of signal transduction. Experiments to identify redox-regulated kinases in the plasma membrane should be carried out.

While pharmacological studies have indicated a role for UV/blue regulated Ca^{2+}-ATPase activity, it is important to identify Ca^{2+}-ATPase activity directly and to biochemically characterise the location and activation properties of the proposed Ca^{2+}-ATPases. In the absence of a biochemical approach, it would be interesting to indirectly determine whether the Ca^{2+}-ATPase is downstream of protein kinase activity, using the method for examining Ca^{2+}-ATPase activity described in this study. While this method for studying the Ca^{2+}-ATPase is far from ideal, it may prove a useful model.

In this study an increase in $[Ca^{2+}]_{cyt}$ in response to UV-A/blue light was identified. However, this response is CRY1-independent. Therefore, these experiments need to be continued in order to identify the CRY1-regulated Ca^{2+} pool involved in the induction of *CHS*. Also, the effect of UV-B light on $[Ca^{2+}]_{cyt}$ was not investigated. Although the proposed UV-B photoreceptor regulating *CHS* in *Arabidopsis* has not been identified, pharmacological inhibitors could be employed to study any UV-B regulated $[Ca^{2+}]_{cyt}$ increase.

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