



<https://theses.gla.ac.uk/>

Theses Digitisation:

<https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/>

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses

<https://theses.gla.ac.uk/>
research-enlighten@glasgow.ac.uk

**UV AND BLUE LIGHT REGULATION OF TRANSCRIPTION
OF THE CHALCONE SYNTHASE GENE IN *ARABIDOPSIS***

A thesis submitted to the University of Glasgow for the degree of
Doctor of Philosophy

William J. Valentine

September, 1998

Division of Biochemistry and Molecular Biology,
Institute of Biomedical and Life Sciences,
University of Glasgow

© William J. Valentine, 1998

ProQuest Number: 10391133

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10391133

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

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

ACKNOWLEDGEMENTS

I am grateful to the BBSRC for the award of a research studentship and for funding this research. Many thanks are due to my supervisor Dr Gareth Jenkins for his input and advice throughout this project. I am also grateful to Dr Bernd Weisshaar at the Max Planck Institute for Plant Breeding in Cologne for his advice the opportunity to study there. Thanks to Ulrike Hartmann for her assistance with the transient expression work in Cologne and donating some of the plasmids for this study. I am grateful to John Christie for his help in establishing the transient expression system and to Helena Wade and Matt Shenton for assistance and advice with the northern blots. Many thanks are due to members of the labs in Glasgow and Cologne (John, Awinder, Jo, Helena, Matt, Sonja, Carol, Bobby, Ulrike, Markus, Katrin, and Ute, not to mention Ruth, John, Paul and Murray at the MPI) for their support during project and ensuring there was always someone who wanted to go for a beer!

I would like to thank Lisa Gallagher, who's love, encouragement and inestimable support have been invaluable over the last three and a bit years. I am indebted to my family for their assistance and support over the last eight years, especially my parents without who's support and encouragement I would not be writing this now. Finally, I would also like to thank everyone I've played football with during my time here for providing a healthy distraction. In particular, the Gazeboes: Andy, Chris, John, Niall and Phil - who'd ever have thought such a bad joke would last so long!

PREFACE

Aspects of the work presented in this thesis have recently been published. The majority of the data presented in Chapter 4 is published in: Hartmann, U., Valentine, W.J., Christie, J.M., Hays, J., Jenkins, G.I. and Weisshaar, B. (1998) Identification of UV/blue light-response elements in the *Arabidopsis thaliana* chalcone synthase promoter using a homologous protoplast transient expression system. *Plant Molecular Biology*, **36**, pg 741 - 754.

CONTENTS

Section	Page
Acknowledgements	i
Preface	ii
Contents	iii
Figures	vii
Abbreviations	xi
Summary	1
References	201
CHAPTER 1: INTRODUCTION	4
1.1 Phytochromes	5
1.2 UV-A/Blue Photoreceptors	11
1.3 UV-B Perception	34
1.4 Signal Transduction	36
1.5 Signal Transduction Mutants	48
1.6 Chalcone Synthase Gene Expression	59
1.7 Transcription Factors Involved In The Regulation of <i>CHS</i>	63
1.8 Conclusions	72
1.9 Project Aims	72
CHAPTER 2: MATERIALS AND METHODS	74
2.1 Materials	74
2.2 General Laboratory Procedures	76

2.3	Plant Material	77
2.4	Illumination of Plant Material	78
2.5	Morphological Measurements	79
2.6	Isolation of Total RNA from Plant Material	81
2.7	Amplification and Preparation of Plasmid DNA	81
2.8	Quantification of DNA and RNA	85
2.9	Gel Electrophoresis	85
2.10	Isolation of DNA Fragments from Agarose Gels	87
2.11	Digestion of DNA with Restriction Endonucleases	88
2.12	DNA Ligations	88
2.13	Klenow 5' Overhang Fill-In of Restriction Fragments	89
2.14	DNA Sequencing	89
2.15	Polymerase Chain Reaction	90
2.16	Northern Blotting	91
2.17	Radiolabelling of DNA	91
2.18	Hybridisation Analysis of Northern Blots	93
2.19	Protoplast Isolation	95
2.20	Transient Gene Expression in Protoplasts	97
2.21	Harvesting of Protoplasts	97
2.22	Measurement of GUS Activity	98
2.23	Measurement of Protein	99

CHAPTER 3:	DEVELOPMENT OF A PROTOPLAST TRANSIENT EXPRESSION SYSTEM IN <i>ARABIDOPSIS</i>	100
3.1	Introduction	100
3.2	Transient Expression In At-glw Protoplasts	104
3.3	Discussion	118

CHAPTER 4:	FUNCTIONAL ANALYSIS OF THE <i>ARABIDOPSIS</i>	
	<i>CHS</i> PROMOTER	120
4.1	Introduction	120
4.2	Function of the LRU in UV/Blue light responsiveness	125
4.3	The Role of the LRU ^{<i>AtCHS</i>} In Light Responsive Promoter Activity	134
4.4	Function of Sequences Upstream of LRU ^{<i>AtCHS</i>} In Light Induction and Quantitative Expression	136
4.5	Discussion	144
CHAPTER 5:	THE ROLE OF CRYPTOCHROMES IN THE	
	CONTROL OF <i>AtCHS</i> REGULATION	148
5.1	Introduction	148
5.2	Overexpression of <i>CRY1</i> Enhances UV-A/Blue Light Induced <i>AtCHS</i> Promoter Activity	149
5.3	Analysis of <i>AtCHS</i> Promoter Activity in Transient Expression Assays Using <i>hy4</i> Protoplasts	153
5.4	Production Of A Cryptochrome Double Mutant	155
5.5	Analysis Of <i>AtCHS</i> Regulation In Cryptochrome Mutants	165
5.6	Discussion	170
CHAPTER 6:	THE ROLE OF SUGAR SENSING IN THE	
	CONTROL OF <i>AtCHS</i> EXPRESSION	174
6.1	Introduction	174
6.2	Sucrose Is Required For <i>AtCHS</i> Light Regulation In Transient Expression Assays	175

6.3	0.5 Mm Sucrose Is Sufficient For <i>AtCHS</i> Light Regulation	176
6.4	<i>AtCHS</i> Is Light Regulated In The Presence Of Glucose	178
6.5	Fructose Affects Light Induced <i>AtCHS</i> Promoter Activity	181
6.6	Light Regulated <i>AtCHS</i> Expression Is Significantly Reduced In The Presence Of 3-O-Methylglucose	183
6.7	<i>AtCHS</i> Expression Is Light Regulated In The Presence Of 2-Deoxyglucose	185
6.8	Mannoheptulose Inhibits Light Regulated <i>AtCHS</i> Expression	187
6.9	Discussion	189
CHAPTER 7:	FINAL DISCUSSION	191
7.1	Introduction	191
7.2	A Homologous Transient Expression System For The Functional Analysis Of <i>Arabidopsis</i> Promoters	191
7.3	Sequence Elements Of The <i>AtCHS</i> Promoter Involved In Light Regulation	193
7.4	UV and Blue Light Photoreceptors Involved In <i>AtCHS</i> Regulation	195
7.5	Sugar Regulation Of <i>AtCHS</i> Promoter Activity	198
7.6	Future Work	199

FIGURES

Figure	Title	Page
1.1	Summary of photomorphogenic mutants in <i>Arabidopsis</i>	50
1.2	Structure of the <i>Arabidopsis</i> <i>CHS</i> promoter	64
2.1	Plasmid DNA used in this study	75
2.2	Spectra of the different light qualities used throughout this study	80
3.1	Photograph of FDA stained At-glw protoplasts	103
3.2	Effects of cell age on <i>AtCHS</i> promoter activity	106
3.3	Effect of different cellulase preparations on <i>AtCHS</i> promoter activity	108
3.4	Effect of cell wall degrading enzyme concentration on <i>AtCHS</i> promoter activity	110
3.5	Effect of heat treatment of cell wall degrading enzyme solution on <i>AtCHS</i> promoter activity	113
3.6	Effect of concentration of heat treated cell wall degrading enzyme solution on <i>AtCHS</i> promoter activity	115
3.7	Kinetics of induction of <i>AtCHS</i> promoter activity in response to HWL	117
3.8	Light regulation of <i>AtCHS</i> promoter activity in At-glw protoplasts	119
4.1	Schematic representation of the <i>AtCHS</i> promoter indicating the sizes of promoter deletions	122
4.2	Transient expression analysis of <i>AtCHS</i> promoter deletion constructs in At-glw protoplasts	123
4.3	Transient expression analysis of <i>AtCHS</i> promoter deletion constructs in At-7 protoplasts	126

4.4	Sequences of MRE _m and ACE _m block mutations and schematic representation of mutated -164 bp promoter constructs	127
4.5	Schematic representation of the cloning procedures used to prepare pAtCHS -164 ACE _m	129
4.6	Schematic representation of the cloning procedures used to prepare pAtCHS -164 dm	130
4.7	Transient expression analysis of -164 bp <i>AtCHS</i> promoter constructs in At-7 protoplasts	132
4.8	Transient expression analysis of -164 bp <i>AtCHS</i> promoter constructs in At-glw protoplasts	133
4.9	Light regulated LRU tetramer promoter activity in At-glw protoplasts	135
4.10	Schematic representation of the cloning procedures used to prepare pAtCHS -1972 dm and pAtCHS -1416 dm	137
4.11	Schematic representation of the cloning procedures used to prepare pAtCHS -668 dm	138
4.12	Schematic representation of the cloning procedures used to prepare pAtCHS -335 dm	139
4.13	Schematic representation of the <i>AtCHS</i> promoter deletion constructs and double mutant constructs	142
4.14	Transient expression analysis of the double mutant constructs in At-glw protoplasts	143
4.15	Schematic representation of constructs for analysis of G-box like element in the -688 bp <i>AtCHS</i> promoter	145
4.16	Transient expression analysis of G-box like element in the -688 bp <i>AtCHS</i> promoter	146
5.1	Schematic representation of constructs for analysis of CRY1 overexpression on light regulation of <i>AtCHS</i> promoter activity	151

5.2	Transient expression analysis of the effect of CRY1 overexpression on the light regulation of <i>AtCHS</i> activity in <i>At-glw</i> protoplasts	152
5.3	Transient expression analysis of the light regulation of <i>AtCHS</i> promoter activity in <i>hy4</i> protoplasts	154
5.4	CRY1 restores the UV-A/blue light induction of <i>AtCHS</i> promoter activity in <i>hy4</i> protoplasts	156
5.5	Hypocotyl extension of cryptochrome mutants	159
5.6	Cotyledon area of cryptochrome mutants	162
5.7	Flowering time of cryptochrome mutants	164
5.8	<i>AtCHS</i> induction in response to different qualities of light in cryptochrome mutants	167
5.9	Effects of a simultaneous UV-B and UV-A/blue light treatment on <i>AtCHS</i> transcript accumulation in cryptochrome mutants	171
6.1	Sucrose is required for light induced <i>AtCHS</i> promoter activity in <i>At-glw</i> protoplasts	177
6.2	Effect of decreasing the concentration of sucrose on <i>AtCHS</i> promoter activity in <i>At-glw</i> protoplasts	179
6.3	Effect of different concentrations of glucose on <i>AtCHS</i> promoter activity in <i>At-glw</i> protoplasts	180
6.4	Effect of different concentrations of fructose on <i>AtCHS</i> promoter activity in <i>At-glw</i> protoplasts	181
6.5	Effect of 3-O-methylglucose on light regulated <i>AtCHS</i> promoter activity in <i>At-glw</i> protoplasts	184
6.6	Effect of 2-deoxyglucose on light regulated <i>AtCHS</i> promoter activity in <i>At-glw</i> protoplasts	186

ABBREVIATIONS

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

At-glw	<i>Arabidopsis</i> photomixotrophic green cell suspension culture
At-7	<i>Arabidopsis</i> dark-grown cell suspension culture
AtCHS	<i>Arabidopsis</i> chalcone synthase
<i>blu</i>	blue light uninhibited
CAB	LHCB1 chlorophyll-binding protein
CFI	chalcone flavone isomerase
CHS	chalcone synthase
<i>cop</i>	constitutive photomorphogenic
cpm	counts per minute
CPRF	common plant regulatory factor
CRY	cryptochrome
CsCl	caesium chloride
DAG	diacylglycerol
DEPC	diethyl pyrocarbonate
<i>det</i>	de-etiolated
DFR	dihydroflavonol reductase
DMSO	dimethyl sulphoxide
ds	double stranded
EGTA	ethyleneglycol-(bis)-(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol

FDA	fluorescein diacetate
<i>fhy</i>	far-red elongated hypocotyl
<i>fnr</i>	ferridoxin NADP+ oxidoreductase
<i>fus</i>	<i>fusca</i>
G _i	inhibitory G protein
GUS	β-glucuronidase
HFR	High fluence response
HRP	horseradish peroxidase
<i>hp</i>	high pigment
<i>hy</i>	long hypocotyl
<i>icx</i>	increased chalcone synthase expression
IP ₃	inositol 1,4,5-trisphosphate
LFR	low fluence response
<i>lh</i>	long hypocotyl
<i>lip</i>	light independent photomorphogenesis
LRU	light regulatory unit
MBP	maltose binding protein
MLCK	myosin light chain kinase
<i>nph</i>	non-phototropic hypocotyl
OAc	acetate
PAA	phenylacetic acid
PAL	phenylalanine ammonia-lyase
PEG	polyethylene glycol
Pfr	form of phytochrome which absorbs maximally far-red light
pH	hydrogen ion concentration (-log ₁₀)
PHY	phytochrome
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PMA	phenylmercuric acetate

PP	protein phosphatase
Pr	form of phytochrome which absorbs maximally red light
PR	pathogenesis-related
psi	pounds per square inch
PVP	polyvinyl pyrrolidone
rbcS	ribulose-1,5-bisphosphate carboxylase small subunit
ROS	reactive oxygen species
35S	cauliflower mosaic virus 35S promoter
SD	standard deviation of the mean
TCH	touch
TEA	tetraethylammonium
UV-A	320-390 nm
UV-B	280-320 nm
UV-C	wavelengths below 280nm
VLFR	very low fluence response
v/v	volume/volume
w/v	weight/volume

SUMMARY

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

In order to identify light responsive *cis*-acting DNA sequence elements of the *Arabidopsis thaliana* chalcone synthase gene (*AtCHS*) concerned with induction by UV-B and UV-A/blue light, we developed a UV/blue light inducible transient expression system. This system involved transfection of chimaeric *AtCHS* promoter constructs into *Arabidopsis* cell culture protoplasts. The reporter enzyme β -glucuronidase (GUS) was used to monitor light responsive promoter activity. Development of this system involved defining and optimising procedures for preparing protoplasts from the *Arabidopsis* cell culture, transfection of chimaeric *AtCHS* promoter constructs into the protoplasts and incubation under defined illumination conditions. An efficient homologous protoplasts transient expression system was developed which subsequently enabled us to undertake functional analysis of the *AtCHS* promoter.

Application of the transient expression system allowed us to define the light responsive *cis*-acting elements concerned with the transcriptional activation of *AtCHS*. This analysis showed that a 1972 bp promoter conferred UV-B and UV-A/blue light

induction of GUS activity. Deletion of the promoter to 164 bp resulted in reduced promoter strength but retention of responsiveness to UV-B and UV-A/blue light. Further deletion abolished transcriptional activity. The 164 bp promoter contains sequences closely resembling the light-responsive unit (LRU^{PcCHS}) of the *Petroselinum crispum* CHS promoter. This *Arabidopsis* CHS promoter region, designated LRU^{AtCHS}, was sufficient to confer UV-B and UV-A/blue light responsiveness to a heterologous core promoter. Mutation of sequences in LRU^{AtCHS} corresponding to the ACGT element and the MYB recognition element of LRU^{PcCHS} resulted in inactivation of 164 bp and 335 bp promoter deletions. However, the mutant 668 bp promoter retained residual UV-B and UV-A/blue light-induced expression, indicating the presence of additional functional sequences upstream of -335. Mutation of a single G-box-like sequence around -442 had no effect on light responsiveness, indicating that it does not function in the light regulation of this promoter. Furthermore, in this analysis no difference in responsiveness to UV-B and UV-A/blue light was observed with any promoter variant tested. This suggests that the UV-B and the UV-A/blue phototransduction pathways regulate transcription factors which interact with common promoter elements. These results are discussed.

In recent years, the genes for two *Arabidopsis* photoreceptors known as cryptochromes, CRY1 and CRY2, have been cloned using molecular techniques (Ahmad and Cashmore, 1993; Ahmad *et al.*, 1998a). The CRY1 photoreceptor has been functionally well defined as a UV-A and blue light photoreceptor and has been shown to be involved in a number of extension growth responses, for instance blue light-mediated inhibition of hypocotyl elongation, and in the control of expression of a number of genes, for example *rbcS* (Jackson and Jenkins, 1995; Conley and Shih, 1995). In contrast, CRY2 is less well characterised having been cloned more recently than CRY1. We employed several different approaches to investigate the role of cryptochromes in the regulation of *AtCHS* promoter activity. Gain of function transient

expression analysis suggests that CRY1 is the primary photoreceptor mediating UV-A/blue light induction of *AtCHS* promoter activity. In addition, northern analysis using various cryptochrome mutant lines, including a *cry1/cry2* double mutant, supports this hypothesis and, furthermore, suggests that (an)other photoreceptor(s) able to respond to UV-A/blue light, exist in *Arabidopsis*. Possible reasons for these observations are discussed.

Physiological, biochemical and molecular studies have demonstrated that various plant genes are subject to regulation by sugars. A well established example is that the genes encoding proteins involved in photosynthesis are subject to negative feedback regulation by sugars accumulated by photosynthetic carbon fixation (reviewed by Graham, 1996; Sheen, 1994). However, metabolic regulation is not restricted to photosynthetic genes. *CHS* gene expression has been shown to be stimulated by sucrose (Moalem-Beno *et al.*, 1997; Tsukaya *et al.*, 1991). Furthermore, Urwin and Jenkins (1997) demonstrated that sucrose, glucose or fructose was required to stimulate *CHS* gene expression in *Phaseolus vulgaris* protoplasts. We used the transient expression system to investigate the role of sugar regulation in the control of *AtCHS* promoter activity in *Arabidopsis* protoplasts. This revealed that sucrose, glucose or fructose was required to stimulate *AtCHS* promoter activity. Furthermore, studies using glucose analogues were performed which suggest that the initial signal for sugar stimulation of *AtCHS* promoter activity is consistent with signalling by hexokinase phosphorylation of hexose sugars. Evidence for this hypothesis is discussed.

CHAPTER 1: INTRODUCTION

In plants, growth and development are governed by complex interactions between environmental signals and endogenous developmental programmes. Of the various environmental signals which control plant development, light is amongst the most important. In addition to utilising light as the energy source for photosynthesis, plants are unique in that light controls numerous developmental and metabolic processes including seed germination, the inhibition of hypocotyl elongation, chloroplast biogenesis, leaf development, anthocyanin formation and flowering (Jenkins, 1991). These light dependent processes are collectively known as photomorphogenesis. In higher plants, photomorphogenesis is controlled by photoreceptors which comprise three classes, each of which is able to detect light of particular wavelengths. These are the phytochromes, which are responsive to red and far-red light, the cryptochromes, which monitor the blue and ultraviolet-A (UV-A) regions of the spectrum, and the ultraviolet-B (UV-B) receptor(s) (Quail, 1994). The light signals perceived by these photoreceptors are transduced via signalling components to bring about the diverse downstream physiological responses associated with photomorphogenic development. The responses regulated in this way have been shown, in many cases, to involve the transcriptional regulation of a variety of both nuclear- and chloroplast-encoded genes (Terzaghi and Cashmore, 1995a).

However, light signals do not act autonomously, but must be integrated with many intrinsic developmental programmes in order to specify temporal and spatial patterns of gene expression and to regulate organelle development and cellular differentiation. Inherently, if a response is controlled by multiple signals, be they environmental, developmental or metabolic, then the regulation of that particular response must be a complex process. This is, perhaps, one of the reasons why our knowledge of the

molecular mechanisms involved in photoreception, signal transduction and transcriptional regulation remains fragmentary.

At present, our knowledge of the phytochrome photoreceptors and signal transduction is still well in advance of our understanding of the corresponding UV/blue perception and signal transduction pathways. However, in recent years, more information has become available on UV/blue photoreception, signal transduction components and transcriptional regulation. A prime example of this is the molecular cloning of the first UV/blue photoreceptor (Ahmad and Cashmore, 1993) which was achieved almost 10 years after the initial cloning of phytochrome. Many likely components of UV/blue light signalling pathways have been identified by several studies. However, in many of these cases, there is little direct evidence to relate these putative signalling events to either specific photoreceptors or to specific responses (Kaufman, 1993; Short and Briggs, 1994; Jenkins *et al.*, 1995). This chapter aims to outline how recent advances made through the application of both biochemical and molecular genetic approaches have contributed to our understanding of photosensory perception, signal transduction and the photoregulation of gene expression in plants.

1.1 PHYTOCHROMES

It is well established that the red/far-red region of the light spectrum is recognised by a class of photoreceptors known as the phytochromes (phy). Indeed, phytochrome has been well studied at the biochemical and molecular genetic levels (Furuya, 1993; Quail *et al.*, 1995). Each of the cloned phytochromes have the same basic structure. They are proteins of approximately 125 kD in size with a single chromophore attachment site in the amino-terminal domain of about 70 kD and an elongated carboxy-terminal domain of around 55 kD. Phytochrome molecules can exist in two photoconvertible forms: the

red light-absorbing Pr form and the far-red absorbing Pfr form. Absorption of red light converts Pr into Pfr and Pfr can be converted back into Pr by illumination with far-red light. Pfr is believed to be the physiologically active form of phytochrome. The interconversion involves a conformational change in the amino-terminal domain of the protein (Quail *et al.*, 1995). Consequently, red-light induced responses mediated by phytochrome are typically reversible by far-red light and this photoreversibility is the classic test for the involvement of phytochrome in a given response.

In most plant species, phytochrome is encoded by a small multigene family. The five *PHY* genes in *Arabidopsis* have been cloned (*PHYA*, *PHYB*, *PHYC*, *PHYD*, *PHYE*) (Clack *et al.*, 1994; Sharrock and Quail, 1989). Each of these genes encodes a protein of 1110 - 1172 amino acids and 124 - 129 kD in size. Each phytochrome molecule carries a covalently linked tetrapyrrole chromophore which is autocatalytically attached via a thiol-ester linkage to a cysteine residue in the amino-terminal chromophore binding domain of the protein (Lagarias and Lagarias, 1989; Quail, 1991). Phytochrome molecules exist as a dimer and are mostly soluble. Dimerization occurs through the carboxy-terminal domain (Edgerton and Jones, 1992; Edgerton and Jones, 1993; Cherry *et al.*, 1993).

Different phytochrome species can be distinguished by their relative abundance and their stability in the Pfr form (Quail, 1994a). For example, *PHYA* is the most abundant phytochrome in etiolated seedlings but is very unstable in the Pfr form. In contrast, *PHYB* is much more stable in the Pfr form and is present in equal amounts in light and dark-grown plants. Therefore, these two phytochromes appear to monitor different aspects of the light environment. In addition, phytochromes are expressed in a developmentally controlled fashion. Much less information for *PHYC*, *PHYD* and *PHYE* is available. However, *phyA/phyB* double mutant plants clearly show residual phytochrome function (Reed *et al.*, 1994).

1.1.1 Phytochrome Responses

Phytochromes regulate a diverse range of responses. They have been separated into three different classes based on their fluence rate requirements (Mancinelli, 1994). These are very low fluence responses (VLFRs), low fluence responses (LFRs) and high irradiance responses (HIRs). VLFRs require as little as $0.1 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ red light for induction. This quantity of light is estimated to convert 0.01% of Pr into Pfr. Interestingly, VLFRs can be induced by far-red treatment alone and therefore do not exhibit classical red/far-red photoreversibility. This is because the Pr form of phytochrome can weakly absorb far-red light (as well as red light) resulting in a small percentage being converted to Pfr. In contrast, LFRs represent the classical phytochrome response. They are activated by red light and, unlike VLFRs, show red/far-red photoreversibility. LFRs require a high amount of Pfr to mediate the response but only for a short time period. It is believed that red light illumination between 1 and $1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ converts approximately 80% of Pr to Pfr whereas subsequent far-red irradiation produces an equilibrium of about 97% Pr and 3% Pfr. Thirdly, HIRs require continuous illumination with relatively high fluence rates, typically greater than $10 \text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and tend to be more efficiently induced by far-red light.

The specific roles of each phytochrome molecule are not yet known. However, transgenic and genetic approaches have begun to reveal how different members of the phytochrome gene family have specialised regulatory roles in controlling VLFRs, LFRs and HIRs under different light qualities.

1.1.2 Functions of Different Phytochromes

A genetic approach has provided important insights into the mechanisms involved in plant photoreception and signal transduction (Chory, 1993; Jenkins *et al.*, 1995). Many photoregulatory mutants have been identified by screening for an elongated hypocotyl

phenotype in white light. Amongst the most important of these mutants are the *hy* (long hypocotyl) mutants of *Arabidopsis* (Koornneef *et al.*, 1980; Chory *et al.*, 1989a). Three of these mutants (*hy1*, *hy2* and *hy6*) are believed to correspond to genes involved with phytochrome chromophore biosynthesis. As such, they are probably deficient in all phytochromes (Parks and Quail, 1991). Parks and Quail (1993) extended these studies by identifying a new class of *Arabidopsis* long hypocotyl mutants. These mutants were isolated by screening for the long hypocotyl phenotype in continuous far-red light. Mutants which also showed an altered ability to respond to red light were discarded because they were likely to be generally defective in phytochrome signalling, for example, mutants in chromophore biosynthesis. The remaining mutants were then designated *hy8* and subsequently shown to contain lesions in the *PHYA* gene (Dehesh *et al.*, 1993).

The nature of the *hy8* phenotype suggests that *PHYA* is required for continuous far-red light perception or far-red HIR. These mutants show altered control of hypocotyl elongation, cotyledon expansion and seed germination suggesting that *PHYA* plays a role in each of these responses (reviewed in Smith, 1995). Further, transgenic plants overexpressing *PHYA* show an enhanced sensitivity to continuous far-red light (McCormac *et al.*, 1993). However, *hy8* shows wild-type responsiveness to continuous red or white light suggesting that *PHYA* plays a considerably less important role under these conditions (Parks and Quail, 1993).

Additional genetic evidence suggests that *PHYB* has a distinct functional role to *PHYA*. In 1993, Reed *et al.* verified that one of the original *hy* mutant loci, *hy3*, encodes the *PHYB* apoprotein. This information has uncovered several roles for *PHYB*. For example, *PHYB* is the principle photoreceptor controlling responses to continuous red light. The *hy3* mutant exhibits extended hypocotyls in continuous white or red light but is unaltered in this response in far-red light (Reed *et al.*, 1993; McCormac *et al.*, 1993). In addition, transgenic *Arabidopsis* plants overexpressing *PHYB* show an

enhanced sensitivity to red light but exhibit wild-type responsiveness to far-red light (McCormac *et al.*, 1993). Mutants lacking PHYB show a phenotype which is altered in shade avoidance responses, flowering time and leaf morphology (reviewed in Smith, 1995).

Aukerman *et al.* (1997) described a null mutation in the *phyD* gene in *Arabidopsis*. This loss of PHYD causes alterations to many of the same shade avoidance responses which are affected in the *phyB* mutant. However, comparison of the two null mutants suggests that PHYB plays a much more prominent role than PHYD in shade avoidance. Mutants lacking PHYC or PHYE have not yet been described and, therefore, their functions have not yet been determined.

1.1.3 Phytochrome Mechanism of Action

The molecular mechanisms by which phytochrome molecules transduce signals remain largely unknown. Analysis of the subcellular localisation of PHYA has provided very little insight into its mechanism of action. PHYA has been clearly shown to be localised in the cytoplasm during darkness but on exposure to light there is a rapid intracellular redistribution resulting in large PHYA cytoplasmic aggregates which are presumably marked for degradation (McCurdy and Pratt, 1986). As well as being found in the cytoplasm, PHYB has been shown to be localised in the nucleus in studies using transgenic *Arabidopsis* plants expressing PHYB-GUS fusion proteins as well as in studies using cell fractionation techniques employing monoclonal antibodies to detect endogenous PHYB (Nagatani and Sakamoto, 1996). This may suggest a direct role for the photoreceptor in mediating gene expression within the nucleus. However, there is no evidence that the phytochrome molecule interacts with DNA in order to mediate its effects on gene expression.

A phytochrome gene identified in the moss *Ceratodon purpureus* has a carboxy-terminal domain homologous to eukaryotic serine/threonine and tyrosine protein kinases (Thummler *et al.*, 1992). This phytochrome exhibited autophosphorylation activity in a red/far-red dependent manner. Therefore it has been postulated to be a light-regulated protein kinase (Algarra *et al.*, 1993). It remains under debate whether phytochrome isolated from higher plants shows kinase activity. However, several sequence similarities to eukaryotic serine/threonine protein kinases and bacterial histidine kinases in the carboxy-terminal of phytochrome have been noted (Thummler *et al.*, 1995).

Mutational analysis has identified a region of PHYA and PHYB molecules which is required for productive interactions with downstream signalling components. A screen for long hypocotyl mutants which contain a mutation either in the *PHYA* or *PHYB* genes but maintains photochemically active phytochrome produced a number of mutant lines. Sequence analysis of these mutants revealed a region of the phytochrome molecule, between amino acids 680 - 840 in both PHYA and PHYB, which appears to be essential for productive interactions with downstream signalling components (Quail *et al.*, 1995; Wagner and Quail, 1995; Xu *et al.*, 1995). As yet, the mechanism of action of phytochrome remains unclear. Evidence of PHYB nuclear localisation, phytochrome as a protein kinase and phytochrome containing a domain involved in triggering signal transduction pathways remains fragmentary. Further study in this area will no doubt soon reveal the genuine mode(s) of action of phytochrome.

1.2 UV-A/BLUE PHOTORECEPTORS

1.2.1 UV-A/Blue Light Responses

Although phytochromes are capable of absorbing light in the UV and blue regions of the spectrum, it is well established that most of the responses of plants to these qualities of light are mediated by separate photoreceptors. These photoreceptors which absorb UV-A (320 - 390 nm) and blue (390 - 500 nm) light are involved in numerous responses including the inhibition of hypocotyl elongation, stomatal opening, phototropism and the transcription of various genes (Kaufman, 1993; Short and Briggs, 1994; Jenkins *et al.*, 1995). It is interesting to note, however, that blue-light mediated responses also exist in organisms other than higher plants. Several studies have shown numerous fungi, algae and bacteria to be responsive to wavelengths of light ranging from 300 - 500 nm. For example, blue light has been shown to regulate gene expression in the photosynthetic bacterium *Rhodobacter sphaeroides* (Shimada *et al.*, 1992). Furthermore, blue light has been shown to regulate morphogenic events in algae (Senger and Schmidt, 1994) and control phototropism in the fungus *Phycomyces* (Galland and Lipson, 1987).

Action spectroscopy has historically played an important role in identifying photoreceptors in many living systems and the action spectra for many UV-A/blue light regulated processes have been established (Briggs and Iino, 1983). Comparison of these action spectra covering a wide range of UV-A/blue light responses reveals considerable variation within the several plants and fungi tested. This observation implies that several different UV-A/blue photoreceptors exist in plants and fungi.

A number of UV-A/blue light responses have action spectra resembling the absorption spectra of flavoproteins. As a result, it has been hypothesised that UV-A/blue light receptors are likely to contain a flavin-type chromophore (Briggs and Iino, 1983; Galland and Senger, 1988a; Short and Briggs, 1994). This hypothesis has been

supported by evidence from biochemical studies performed using compounds which interact with flavins (Briggs and Iino, 1983; Galland and Senger, 1988a; Warpeha *et al.*, 1992). However, several action spectra cannot be easily explained by this single flavin chromophore hypothesis. Further studies have indicated that pterins can function as either the sole chromophore pigment or as an accompanying chromophore pigment for UV-A/blue photoreception (Galland and Senger, 1988b; Schmidt *et al.*, 1990).

The variability observed in action spectra for UV-A/blue light-mediated responses in plants could well be explained by the presence of several different chromophore types and their possible combinations. Identification and analysis of the first UV-A/blue light photoreceptor has indicated that this may indeed be the case. CRY1 is thought to have flavin adenine dinucleotide (FAD) as one chromophore and, at least when the CRY1 protein is heterologously produced in *E. coli*, has the pterin methenyltetrahydrofolate (MTHF) as the other (Ahmad and Cashmore, 1996; Malhotra *et al.* 1995).

1.2.2 The CRY1 Photoreceptor

One of the major milestones in the field of blue light perception was the first isolation of a blue light photoreceptor (Ahmad and Cashmore, 1996). This photoreceptor was designated CRY1 (short for cryptochrome) and was isolated using molecular genetic techniques from *Arabidopsis thaliana*. As with many important insights into the mechanisms involved in plant photoreception and signal transduction (Chory, 1993; Jenkins *et al.*, 1995), CRY1 was identified by the analysis of photoregulatory mutants.

1.2.2.1 Arabidopsis Blue Light Mutants

The *hy* mutants of *Arabidopsis* were isolated by screening a mutagenised population for plants with a long hypocotyl, lacking normal inhibition of hypocotyl growth, when grown under white light (Koornneef *et al.*, 1980). Several mutants which were

identified in this way were later found to be deficient in certain phytochrome molecules (Chory, 1992; Chory, 1993; Whitelam and Harberd, 1994). However, the *hy4* mutant plants showed normal inhibition of hypocotyl elongation when grown under red and far-red light, suggesting that these mutants respond normally to phytochrome-mediated signals. In contrast, when *hy4* plants were grown under blue light, they showed a greatly reduced inhibition of hypocotyl elongation (Koornneef *et al.*, 1980; Ahmad and Cashmore, 1993). This observation implies that *hy4* mutants are specifically altered in blue light perception. In addition, further studies have revealed that *hy4* mutants also exhibit a longer hypocotyl phenotype under UV-A and green light qualities (Lin *et al.*, 1995a). Furthermore, it has subsequently been demonstrated that *hy4* mutants have reduced induction of flavonoid biosynthesis gene expression and anthocyanin synthesis in blue light (Jackson and Jenkins, 1995; Ahmad *et al.*, 1995).

Several additional *Arabidopsis* mutants were subsequently identified by screening a mutagenised population for long hypocotyls under blue light (Liscum and Hangarter, 1991). Three of these mutants exhibited no blue light-mediated inhibition of hypocotyl elongation and were designated *blu1*, *blu2* and *blu3* (blue light uninhibited). However, subsequent genetic analysis of these mutants has revealed that the *blu* mutants are, in fact, alleles of *hy4* (Ahmad and Cashmore, 1996).

1.2.2.2 Cloning of the *HY4* locus

The *HY4* gene was isolated using a T-DNA tagged allele of the *Arabidopsis hy4* mutant (Ahmad and Cashmore, 1993). The protein encoded by the *HY4* gene was found to be 681 amino acids in length and approximately 75 kD in size. This amino acid sequence, upon examination, was found to bear striking sequence similarity to the DNA photolyase family of proteins (Ahmad and Cashmore, 1993). DNA photolyase proteins are a class of flavoproteins which catalyse the light-dependent repair of pyrimidine dimers generated by exposure of DNA to UV irradiation (Sancar, 1994). It was,

however, considered unlikely that *HY4* encoded an actual photolyase because a conserved tryptophan residue implicated in specific recognition of pyrimidine dimers during photolyase function was not conserved in the *HY4* sequence. In addition, the *HY4* amino acid sequence contained a C-terminal extension of approximately 200 amino acids beyond the region of photolyase homology. This region shows similarity to rat smooth-muscle tropomyosin A and is not found in photolyases (Ahmad and Cashmore, 1993). Subsequent genetic and photobiological studies provided convincing evidence that the *Arabidopsis* *HY4* gene encoded the photoreceptor mediating the blue light-dependent *hy* response and Cashmore and co-workers later re-named the *HY4* protein as cryptochrome 1 (*CRY1*), after the term previously used to describe plant and fungal blue light photoreceptors (Ahmad and Cashmore, 1996).

1.2.2.3 DNA photolyases

The characterisation of *CRY1* has been greatly facilitated by the extensive studies performed on microbial DNA photolyases (Sancar, 1994). As previously mentioned, these enzymes catalyse the blue light-dependent repair of pyrimidine dimers in DNA damaged by UV light in a process known as photoreactivation. There are two types of photolyase which both absorb light energy by the action of a primary light-harvesting chromophore bound non-covalently at the N-terminal of the protein. Short wavelength type photolyases (absorbing maximally at 350-370 nm) bind a pterin as the primary light-harvesting chromophore whereas the long wavelength type (absorbing maximally at 450 nm) bind a deazaflavin. In each case, light energy is transferred to a second chromophore, flavin adenine nucleotide (FAD), bound non-covalently at the C-terminal of the enzyme. The photoreduced FAD molecule can then provide the energy required for the cleavage of the pyrimidine dimer. It is generally considered that both chromophores are required for DNA repair although light absorption by either the deazaflavin/pterin or FAD molecule can result in the excision of pyrimidine dimers

(Jorns *et al.*, 1990). This knowledge of photolyases may provide a valuable insight into the mechanism of action of CRY1 and other plant UV-A/blue photoreceptors.

The N-terminal two-thirds of CRY1 shows strong sequence similarity to microbial DNA photolyase. Over this region, the similarity between CRY1 and the photolyases is as high as the similarity between the photolyases themselves. Furthermore, it is interesting to note that this sequence homology was highest in regions known to be involved in photolyase chromophore binding (Ahmad and Cashmore, 1993).

1.2.2.4 The CRY1 FAD Chromophore

Lin and co-workers, in 1995, purified baculovirus-expressed CRY1 from insect cells. Purified CRY1 is a yellow protein with an absorption spectrum which resembles that of a flavoprotein. The purified protein had no apparent photolyase activity *in vitro* and expression of CRY1 could not rescue a photolyase deficient mutant of *E. coli*. These observations are consistent with the predictions from the initial sequence analysis that CRY1 is a non-photolyase, flavin-type photoreceptor.

It was also shown that recombinant CRY1 binds non-covalently to stoichiometric amounts of oxidised flavin adenine dinucleotide (FAD). Detailed redox studies of the holoprotein indicated that the chromophore can exist in a flavosemiquinone form (FADH). Under anaerobic conditions, the semiquinone form of the flavin was surprisingly stable relative to the analogous flavin bound to *E. coli* photolyase. This flavosemiquinone form (FADH) absorbs green light (500 - 600 nm.). Therefore, CRY1 may be capable of responding to blue, UV-A and green light and this could be explained by the bound flavin switching between different redox states (Lin *et al.*, 1995b). This is consistent with the observation that transgenic tobacco plants overexpressing CRY1 exhibit much shorter hypocotyls in UV-A, blue and green light than non-overexpressing control plants (Lin *et al.*, 1995a). The degree of

hypersensitivity demonstrated by these plants under these light conditions correlated with the level of overexpression of CRY1.

1.2.2.5 CRY1 MTHF Chromophore

The amino acid sequence of the more N-terminal chromophore-binding domain of CRY1 shows greater sequence relatedness to the long wavelength class of photolyases that bind deazaflavin as a second chromophore (Sancar, 1994). However, Malhotra *et al.* (1995) provided evidence that the second or more N-terminal chromophore of CRY1 may well be the pterin methenyltetrahydrofolate (MTHF) as opposed to the deazaflavin previously suggested. The N-terminal part of CRY1 (the "photolyase" domain) was expressed as a fusion with maltose-binding protein in *E. coli*. Spectroscopic analysis of this fusion protein revealed that it contained two chromophores which were identified as FAD and MTHF. Consistent with the findings of Lin *et al.* (1995b), the MBP-HY4 (MBP-CRY1) fusion showed no apparent photolyase activity in these studies. However, the precise nature of the CRY1 second chromophore in *Arabidopsis* plants, as opposed to *E. coli* cells, remains to be determined.

1.2.2.6 CRY1 Mode of Action

In 1995, Ahmad *et al.* performed mutational studies in order to identify those regions of the protein which are functionally important. EMS mutagenesis was performed in order to generate mutant alleles of *hy4* and over twenty such alleles were characterised. Many of these alleles were point mutations within the region similar to photolyases and thought to be involved in chromophore binding. Interestingly, however, seven point mutations were identified within the C-terminal region which shows similarity to rat smooth-muscle tropomyosin A. This observation indicates that this region was also important for CRY1 function. Moreover, three of these mutations were conservative

arginine to lysine substitutions which suggests that there are conformational constraints in this domain of the protein.

A mode of action for CRY1 has been suggested which is based on our present knowledge of the photolyase-like region and takes into account the importance of the C-terminal domain. This model suggests that CRY1, like photolyase, mediates a light-dependent redox reaction and the redox signal, in the form of an electron, is then transferred to a "downstream partner" which is activated by this process. According to this model, the CRY1 C-terminal domain would be involved in binding the "downstream partner" (Cashmore, 1997). Indeed, redox activity has been implicated in several blue light responses in other species (Jenkins *et al.*, 1995). The hydrophobicity plot of CRY1 reveals no obvious membrane-spanning domains which suggests that the protein is, like phytochrome, soluble (Ahmad and Cashmore, 1993). Subsequently, immunological studies confirmed that CRY1 is, indeed, cytosolic and northern analysis demonstrated that *HY4* gene expression is not light-regulated and is expressed in stems, leaves, flowers, siliques and to a lesser extent in roots (Lin *et al.*, 1996).

1.2.2.7 CRY1-Mediated Responses

Several studies have implicated CRY1 in a number of UV-A/blue light-dependent responses. As previously mentioned, mutants in the *HY4* gene are impaired in the inhibition of hypocotyl extension by UV-A, blue and green light (Ahmad *et al.*, 1995). In addition, Jackson and Jenkins (1995) reported that CRY1 is involved in a variety of extension-growth responses. They demonstrated that *hy4* mutant plants have longer bolted stems, increased petiole length and increased leaf width and area in blue light. Furthermore, it has been shown that apical hook opening is also controlled by CRY1 (Liscum and Hangarter, 1993).

CRY1 has also been shown to be involved in a number of gene expression responses. For instance, northern analysis revealed that *hy4* mutants have a reduction in transcripts encoding chalcone synthase and other flavonoid biosynthesis genes (Jackson and Jenkins, 1995). It has also been shown that *hy4* mutants have reduced anthocyanin synthesis in blue light (Jackson and Jenkins, 1995; Ahmad *et al.*, 1995). CRY1 is also required for the full expression of certain nuclear-encoded chloroplast protein genes, including ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS*) (Conley and Shih, 1995). Thus CRY1 appears to be concerned with the perception of blue light in a range of gene expression and extension growth responses.

It is noteworthy, however, that CRY1 is not involved in all UV-A/blue responses. For example, the low fluence blue light-induced expression of genes encoding the LHCB1 chlorophyll-binding protein (*CAB*) appears to be unaltered in dark-grown *hy4* seedlings (Gao and Kaufman, 1994). Further, Lin and co-workers (1996) observed that *hy4* mutants show normal phototropism. These observations imply that CRY1 is not the only UV-A/blue light photoreceptor in *Arabidopsis*.

1.2.2.8 CRY1-Related Sequences

CRY1 related sequences have been identified in tomato, pea and rice (Ahmad and Cashmore, 1996). Recently, a sequence with 49% amino acid sequence identity to CRY1 in the first 500 amino acids has been identified in *Chlamydomonas reinhardtii*, mapping to a site on the genome distinct from that encoding photolyase activity (Small *et al.*, 1995). This amino-terminal sequence has greater similarity to CRY1 than to any microbial photolyases, and moreover lacks the conserved Trp-277. The *Chlamydomonas* gene, named *CPH1*, has in addition a 300 amino acid C-terminal extension with no homology to that of either CRY1 or CRY2 and thereby almost certainly represents another cryptochrome photoreceptor of the CRY-gene family. This

type of photoreceptor is evolutionarily quite ancient. Whether similar photoreceptors exist in non-plant organisms remains to be determined.

1.2.3 The CRY-2 Photoreceptor

Ahmad and Cashmore (1996) performed Southern analysis and screened cDNA and genomic libraries to reveal a CRY1-related sequence in the *Arabidopsis* genome which they named *CRY2*. The sequence of this gene was published in electronic form (Lin *et al.*, 1996). The amino acid sequence encoded by the *CRY2* gene has extensive homology to *CRY1* in the amino terminal photolyase-like domain (58% identity over 490 residues). It is this region of the protein that encodes the chromophore-binding domain. The carboxy-terminal 120 amino acids of *CRY2* is, however, very different from the corresponding region of 186 residues of *CRY1* (Lin *et al.*, 1998).

Whilst the *CRY2* sequence, like *CRY1*, shows considerable similarity to *E. coli* photolyase around the flavin-binding site (Park *et al.*, 1995), recombinant *Arabidopsis* *CRY2* protein expressed in and purified from insect cells shows no photolyase activity and contains flavin adenine dinucleotide (Lin *et al.*, 1998). These observations are consistent with the presence of a highly conserved flavin-binding domain in *CRY2* and the absence, at the corresponding position, of a critical tryptophan residue which, in *E. coli* photolyase, is required for DNA binding and photolyase activity (Li and Sancar, 1990; Kim *et al.*, 1992).

1.2.3.1 Expression of *CRY2* is Negatively Regulated by Blue Light

Western analysis of *Arabidopsis* extracts using polyclonal antibodies prepared against the carboxy-terminal domain of *CRY2* revealed that *CRY2* is a soluble protein and is expressed in hypocotyls, cotyledons, stems, leaves, roots and flowers (Lin *et al.*, 1998). This is very similar to the pattern of expression of *CRY1* which was shown,

again using polyclonal antibodies, also to be a soluble protein expressed in stems, leaves, roots and flowers (Lin *et al.*, 1996).

However, further analysis revealed that the expression of CRY2 is negatively regulated by blue light whereas the relative abundance of CRY1 remains unchanged. Interestingly, levels of the CRY2 protein also declined dramatically under UV-A and green light which, similarly, had no effect on levels of CRY1 expression (Lin *et al.*, 1998). Previous studies have shown that blue, UV-A and green light comprise the broad action spectrum of CRY1 (Lin *et al.*, 1995b; Young *et al.*, 1992). It appears unlikely, however, that CRY1 is involved in regulating the expression of CRY2. This is because western blots showed that CRY2 expression is unaltered in *hy4* mutant plants which do not express detectable levels of CRY1 (Lin *et al.*, 1998).

The down-regulation of CRY2 protein levels in response to blue light is a rapid one. Levels of CRY2 reach steady state within one hour after exposure of etiolated seedlings to blue light. Very similar changes in CRY2 abundance were observed in response to blue light with red-light-grown seedlings (Lin *et al.*, 1998). This observation confirms the wavelength specificity of CRY2 regulation.

Interestingly, northern analysis revealed that the blue light-induced decrease in CRY2 levels is not explained by changes in transcript abundance. There was little change in the amount of CRY2 mRNA when plants grown in red light were exposed to blue light for one hour. However, a rapid decline of CRY2 protein abundance was observed in the plants (Lin *et al.*, 1998). These observations indicate that the negative regulation of CRY2 in blue light is not mediated at the level of transcription or mRNA stability. It remains to be seen whether CRY2 is regulated by a protein degradation system and, indeed, whether CRY2 negatively autoregulates its own expression.

1.2.3.2 CRY1 and CRY2 Overlap in Function

Ahmad *et al.* (1998) overexpressed CRY1 and CRY2 fusion proteins in transgenic *Arabidopsis* plants. A range of fusion proteins were used including the CRY1 N-terminal domain (chromophore binding) fused to the CRY2 C-terminal domain and the CRY2 N-terminal domain fused to the C-terminal domain of CRY1 (thought to be involved in signalling or protein:protein interactions). The fusion constructs were tested in *hy4* mutant plants for their ability to restore blue light-dependent inhibition of hypocotyl elongation. Transgenic plants overexpressing such fusion constructs as well as those overexpressing intact CRY1 and CRY2 photoreceptors demonstrated that all of the constructs tested mediated blue light-dependent inhibition of hypocotyl elongation to varying degrees. In addition, these overexpressing plants showed increased anthocyanin production compared to *hy4* mutants. These observations suggest that CRY1 and CRY2 share considerable similarity in both their specificity and mode of action.

Given that the N-terminal domains of CRY1 and CRY2, which are thought to be involved in chromophore binding, are so similar (58% identity at amino acid level) it is perhaps unsurprising that these domains were functionally interchangeable in this study. In contrast, the C-terminal domains of CRY1 and CRY2 appear to be different both in size and amino acid composition and yet these domains were also shown to be functionally interchangeable in the various fusion constructs. It appears that CRY1 and CRY2 share considerable overlap in function and that these identified domains are, at least to some degree, interchangeable.

Interestingly, this study did not reveal whether CRY2 protein instability could be assigned to any specific domain. All of the fusion proteins tested showed a light-labile phenotype. This observation was in contrast to intact CRY1 which showed unaltered levels of protein expression under all of the light qualities tested. The possibility exists

that there is no discrete domain within the protein responsible for its stability or there may be multiple regions throughout the protein required for stability.

Northern analysis showed that the CRY2 photoreceptor shows greater stability under low fluences (less than $1 \text{ mmol.m}^{-2}.\text{s}^{-1}$) of blue light (Ahmad *et al.*, 1998). It has been suggested that CRY2 might undergo a light-dependent conformational change which acts as a possible trigger for ubiquitin-dependent protein degradation.

1.2.3.3 CRY2 Responses

CRY2 has been demonstrated to play a role in de-etiolation of *Arabidopsis* seedlings. CRY2 overexpressing transgenic *Arabidopsis* plants developed significantly shorter hypocotyls and larger cotyledons than wild-type plants when grown under blue light over a range of fluence rates (Lin *et al.*, 1998). The shorter hypocotyl phenotype of the overexpressing plants is likely to reflect a hypersensitive response due to elevated levels of CRY2 protein. Similar observations have been made previously for CRY1 (Lin *et al.*, 1995a; Lin *et al.*, 1996). It is worth noting that *hy4* null mutants grown under relatively high fluence rates of blue light had hypocotyls almost as long (80 - 90%) as those of etiolated wild-type seedlings (Ahmad and Cashmore, 1993; Koornneef *et al.*, 1980; Lin *et al.*, 1996). In addition, plants overexpressing CRY1 show greater inhibition of hypocotyl elongation than plants overexpressing CRY2 when grown under fluence rates of blue light over $1 \text{ } \mu\text{mol.m}^{-2}.\text{s}^{-1}$. When the intensity of light is less than $1 \text{ } \mu\text{mol.m}^{-2}.\text{s}^{-1}$, however, CRY2 overexpressing plants have shorter hypocotyls than CRY1 overexpressing plants (Lin *et al.*, 1998). This suggests that CRY1 is the predominant photoreceptor mediating hypocotyl inhibition under a relatively high fluence rate of blue light whereas CRY2 may function in de-etiolation under low fluence rates of blue light. This hypothesis was partly supported by studies using null mutants of *cry2*. These mutant seedlings grew much taller than wild-type seedlings under low fluence rates of blue light (lower than $10 \text{ } \mu\text{mol.m}^{-2}.\text{s}^{-1}$) and this difference was greatly

reduced under high fluence rates of blue light. In contrast, *hy4* null mutants showed considerably longer hypocotyls than wild-type under high fluence rate blue light as expected but also exhibited a long hypocotyl phenotype under low fluence rates of blue light (Ahmad *et al.*, 1998).

Interestingly, CRY2 also appears to play a role in cotyledon development. It was observed that the cotyledons of *cry2* mutant plants opened later than those of wild-type seedlings when grown under low fluence rate blue light. Furthermore, transgenic plants overexpressing CRY2, when grown under blue light, were seen to have larger cotyledons than wild-type plants (Lin *et al.*, 1998). It appears that the function of CRY2 in early photomorphogenesis is, for the most part, restricted to low fluence rates of blue light. Under low fluence rate light, both CRY1 and CRY2 contribute to the control of hypocotyl elongation and cotyledon opening whereas under higher fluence rates, CRY1 seems to predominantly mediate these responses. Perhaps this is the basis for a mechanism which maximises sensitivity to low fluence rates of blue light.

CRY2 has also been shown to be involved in the regulation of flowering time in *Arabidopsis* (Guo *et al.*, 1998). Using null mutants of *cry2*, it was shown that mutant plants flower later than wild-type. Further analysis revealed that the difference in flowering time between *cry2* mutants and wild-type was greater under long-day conditions. Indeed, under short day photoperiods, *cry2* mutant plants flowered earlier than wild-type. It appears that the *cry2* mutation results in a partial loss of photoperiodic regulation of flowering time. These observations were verified when *cry2* was discovered to be allelic to a previously characterised late flowering mutant designated *fha1* (Guo *et al.*, 1998). Koornneef *et al.* (1991) screened EMS treated or irradiated populations to find late flowering mutants under long day conditions. This screen was designed to study flowering and allow the production of a model for floral initiation in *Arabidopsis*. They produced 42 mutants which represented 11 different loci. One group of mutants from this study was designated *fha* (1, 2 and 3) and they showed

increased numbers of rosette and cauline leaves and flowered later than wild-type under long-day conditions.

The CONSTANS (CO) gene encodes a GATA-1-type transcriptional regulator which is required for the accelerated flowering of *Arabidopsis* under long-day conditions (Puterill *et al.*, 1995). CO has been proposed to be a downstream component in a signalling pathway from a hypothesised blue light receptor (Coupland, 1995). Northern analysis was used to study CO mRNA levels in *cry2* mutant plants grown under different photoperiods. Under long-day conditions, *cry2* mutant plants showed CO mRNA levels three times lower than wild-type whereas under short-day photoperiods the amount of CO message was only slightly reduced. These observations perhaps explain, at least in part, the altered regulation of flowering time seen in *Arabidopsis cry2* mutants.

Another interesting aspect of CO expression is that there is a two-fold increase of CO mRNA in the *hy1* mutant which is impaired in the biosynthesis of the phytochrome chromophore (Simon *et al.*, 1996) and activity of CO is required for the early flowering phenotype of *hy1* and *phyB* mutants (Puterill *et al.*, 1995). It appears that CRY2 and phytochrome function antagonistically to regulate gene expression in the case of CO.

The CRY2 photoreceptor has also been implicated in phototropism. Mutant plants lacking both CRY1 and CRY2 photoreceptors are impaired in the first positive phototropism response (Ahmad *et al.*, 1998). Furthermore, transgenic plants overexpressing CRY2 showed enhanced phototropic curvature. Thus, it appears that CRY2 plays a role in the phototropic response as well as the inhibition of hypocotyl elongation, cotyledon opening and expansion and the regulation of flowering time in *Arabidopsis*.

1.2.4 Additional Cryptochrome Photoreceptors

Sequence analysis has revealed CRY2 to be almost identical (89% identity) to a gene, named SA-phr1, from *Sinapis alba* (Batschauer, 1993). This mustard gene was isolated by screening a cDNA library using a set of oligonucleotides with degenerate sequences corresponding to a highly conserved region in the carboxy-terminal of microbial photolyases. The SA-phr1 gene was originally thought to encode a photolyase as it was found to complement, albeit weakly, a photolyase-deficient mutant of *E. coli*.

More recently, however, Malhotra *et al.* (1995) expressed SA-phr1 as a maltose binding protein (MBP) fusion in *E. coli* and showed that it had no photolyase activity *in vitro*. In addition, the SA-phr1 gene lacks a conserved tryptophan residue, Trp-277 in *E. coli*, found in all microbial photolyases but not in CRY1 or CRY2. These observations suggest that SA-phr1 is not, as first suspected, a photolyase. In fact, the *in vitro* absorption properties of the MBP-SA-phr1 fusion resemble those of a MBP-CRY1 fusion, suggesting an action spectrum with peaks in the blue region (Malhotra *et al.*, 1995). This is in contrast to the action spectrum of photolyase activity in *Arabidopsis* (a close relative of *Sinapis*) which peaks around 360-380 nm with very little activity in the blue region (Pang and Hays, 1991). These observations indicate that SA-phr1 may well be a cryptochrome-like photoreceptor. However, the SA-phr1 sequence lacks the C-terminal extensions found in CRY1 and CRY2 and preliminary immunolocalisation studies suggest that most of the SA-phr1 protein is located in the nucleus (Batschauer, 1993). Whether SA-phr1 represents an additional cryptochrome photoreceptor remains to be seen.

Small *et al.* (1995) have isolated a gene from *Chlamydomonas reinhardtii* with 49% identity to CRY1 over the first 500 amino acids. This gene maps to a site on the genome distinct from that encoding photolyase activity and the amino-terminal part shows greater similarity to CRY1 than to any of the microbial photolyases. In addition, this

sequence lacks the conserved tryptophan residue of photolyases. This gene, named *CPH1*, has a carboxy-terminal extension of approximately 300 amino acids which shows no similarity to CRY1 or CRY2. This gene is a good candidate to be an additional cryptochrome photoreceptor and, as such, would suggest that this type of photoreceptor is evolutionarily quite ancient.

Similarly, Wada *et al.*(1996) have identified five CRY1-like sequences in the fern *Adiantum capillus-veneris*. Whilst several blue light responses have been documented in this species (Wada and Sugai, 1994) no functions have yet been assigned to these five CRY1-like sequences.

In addition, CRY1-related sequences have been identified in tomato, pea and rice (Ahmad and Cashmore, 1996). However, the important possibility exists that there are additional blue light receptors in higher plants which are unrelated to CRY1. There may be distinct flavin-type blue-light photoreceptors, but there is some physiological evidence for the possible involvement of a carotenoid (zeaxanthin) in phototropism and guard cell opening (Quiñones and Zieger, 1994; Srivastava and Zieger, 1995). Opsins have also recently been proposed as photoreceptor pigments in higher plants (Lorenzi *et al.*, 1994). These studies, though not conclusive, are worthy of consideration bearing in mind the limitations of action spectra and inhibitor studies. There appear to be, in addition, distinct photoreceptors absorbing in the UV-A and UV-B regions of the spectrum (Ballare *et al.*, 1995; Young *et al.*, 1992). It is therefore a possibility that these additional "cryptochromes", with no sequence relatedness and entirely different chromophore composition to CRY1, may exist in higher plants.

1.2.5 Cryptochrome and Phytochrome Interactions

Early genetic analysis using double mutant plants, which are lacking both phytochromes and CRY1, supported the notion that blue light and phytochrome

photosensory systems are, at least in part, distinct. Double mutants made between *hy4* and the phytochrome-deficient mutants, *hy1*, *hy2* and *hy6*, exhibit a hypocotyl length that is the sum of the corresponding single mutant phenotypes (Koornneef *et al.*, 1980). Furthermore, plants homozygous for *blu1* (*hy4*) and *hy6* have very long hypocotyls and closed cotyledons characteristic of etiolated (dark-grown) seedlings, suggesting that most, if not all, de-etiolation responses are mediated by either phytochrome and/or CRY1 (Liscum and Hangarter, 1991).

However, more recent evidence suggests that, at least for some responses, CRY1 shows a functional dependence on phytochrome A or phytochrome B in *Arabidopsis*. Ahmad and Cashmore (1997) demonstrated that the CRY1-mediated inhibition of hypocotyl elongation can be substantially increased by increasing the amount of active phytochrome by treating plants with a pulse of low-fluence rate red light after blue light treatments. Similarly, equivalent treatment with pulses of far-red light significantly decreased the effectiveness of CRY1-mediated hypocotyl elongation. Furthermore, analysis of severely phytochrome-deficient mutants showed that CRY1-mediated blue-light responses were considerably reduced, even though western blots confirmed that levels of CRY1 photoreceptor are unaltered in these mutant backgrounds. The authors suggest that CRY1-mediated inhibition of hypocotyl elongation and anthocyanin production requires active phytochrome for full expression and that this requirement can be supplied by low levels of either PHYA or PHYB.

1.2.6 A Blue Light Photoreceptor for Phototropism

Phototropism is the adaptive process by which plant organs directionally bend in response to a light source in order to optimise light capture for photosynthesis. 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 a few species, including *Arabidopsis* (Steinitz *et al.*, 1985). Studies

have shown there to be a considerable degree of complexity in the physiological bending response to blue light which can be divided into two phases, first and second positive curvature, depending on their fluence and time requirements (Firm, 1994). Whilst much of our present information about phototropism and its complexity has come from fluence-response measurements (e.g. Konjevic *et al.*, 1989; Konjevic *et al.*, 1992), perhaps a greater understanding has come from *Arabidopsis* mutants altered in phototropic responsiveness (Liscum and Hangarter, 1994; Liscum and Briggs, 1995).

1.2.6.1 The *nph* Mutants

In 1995, Liscum and Briggs performed a screen designed to detect phototropic null mutants. *Arabidopsis* seeds were mutagenised using fast neutrons and the M₂ seedlings were screened for mutants altered in second-positive phototropism which occurs on exposure to prolonged irradiation in a time-dependent manner. As a result, they found mutations in four different loci associated with phototropism. These mutants were designated *nph1* to *nph4* (for non-phototropic hypocotyl).

The *nph1* null alleles were shown to lack detectable levels of a phosphoprotein involved in a blue light-inducible phosphorylation reaction at the plasma membrane in wild-type plants (see 1.2.6.2). In *nph2*, *nph3* and *nph4*, although phototropism was impaired, normal amounts of the phosphoprotein were present and light-inducible phosphorylation was similar to that found in wild-type plants. The *nph2* and *nph3* mutants could not be distinguished by their phenotype although they were genetically independent. The *nph4* mutant was unique in that it was impaired in both gravitropism and phototropism and thus appeared to be altered in some function shared by both of these responses. For these reasons, Liscum and Briggs (1995) ordered these genes in the signal transduction pathway as *nph1* --> *nph2*, *nph3* --> *nph4*.

1.2.6.2 The Blue Light-Dependent Phosphorylation Reaction

In 1990, Short and Briggs observed that illumination with blue light of isolated pea membranes caused an increase in phosphorylation of a 120 kD protein by exogenous [$\gamma^{32}\text{P}$] ATP. Furthermore, they showed that pre-illumination of the tissue with blue light before isolation of the membranes strongly reduced the effectiveness of the *in vitro* irradiation. Subsequent studies went on to reveal that when etiolated tissue was pre-incubated with inorganic ^{32}P before illumination, in order to allow synthesis of endogenous radiolabelled ATP, an increase in phosphorylation was detected rather than a decrease (Short *et al.*, 1992). The authors explained these observations by proposing that blue light somehow causes sites in the 120 kD protein to become exposed and subsequently these sites are phosphorylated by endogenous ATP. This model suggests that when the tissue is irradiated *in vivo*, these sites are unavailable for phosphorylation by exogenous [$\gamma^{32}\text{P}$] ATP. However, when irradiation occurs *in vitro* no endogenous ATP is present and as a result the exposed sites are rapidly phosphorylated by exogenous [$\gamma^{32}\text{P}$] ATP. Thus it became apparent that, at least in pea, a 120 kD protein is phosphorylated in response to blue light.

Other studies have revealed that similar blue light-induced phosphorylation effects in several other plant species including maize (Hagar *et al.*, 1992; Palmer *et al.*, 1993), oat (Salomon *et al.*, 1996) and *Arabidopsis* (Reymond *et al.*, 1992a). In each of these cases, the phosphorylated polypeptide is between 114 and 130 kD in size and the phosphorylation response is very rapid requiring less than one second of illumination.

Interestingly, Short and co-workers (1992) showed that purified plasma membrane fractions retain their phosphorylation response even when partially solubilised by detergent indicating that the photoreceptor, kinase and substrate are all located in the membrane fraction. In addition, the 120 kD protein from pea contains a nucleotide binding site and may have an inherent kinase activity and the capacity for autophosphorylation (Short *et al.*, 1993). Indeed, it has been suggested that both the

photoreceptor and kinase activities are both mediated by the same polypeptide (Short and Briggs, 1994).

1.2.6.3 Blue Light-Dependent Phosphorylation and Phototropism

Several photobiological correlations have been made between the blue light-dependent phosphorylation reaction and phototropism. Short and Briggs (1990) showed that the fluence rate and time dependence of blue light-induced phosphorylation are compatible with first-positive phototropism. In addition, tissue localisation of the 120 kD protein is consistent with that predicted for the photoreceptor for first-positive phototropism. Similarly, the light-induced phosphorylation of a 114 kD protein has been correlated with phototropism in maize (Palmer *et al.*, 1993). Furthermore, it has been shown that following unilateral irradiation with blue light, the degree of phosphorylation of a 116 kD protein is significantly higher at the irradiated side than at the shaded side of oat coleoptile tips (Salomon *et al.*, 1996). In addition, Short *et al.* (1992) showed that potassium iodide (KI), sodium azide (NaN_3) and phenylacetic acid (PAA), at equivalent concentrations used to inhibit phototropism, reduce blue light-induced phosphorylation in pea membranes. Taken together these observations suggest that the blue light induced phosphorylation reaction plays a role in phototropism.

1.2.6.4 A Role for NPH-1 in Phototropism

It was demonstrated by Reymond *et al.* (1992a) that blue light induces phosphorylation of a 124 kD plasma membrane protein in *Arabidopsis*. Indeed, in collaboration with the Poff laboratory, they went on to show that the phototropic mutant JK224 was defective in the blue light-dependent phosphorylation reaction (Reymond *et al.*, 1992b). This was the first piece of genetic evidence which linked the phosphoprotein to first positive phototropism. As previously mentioned, Liscum and Briggs (1995) have extended this work with the *nph* mutants and have shown that JK224 was an allele of *nph1*.

Interestingly, *nph1-2* (JK224) etiolated seedlings were impaired only in first positive phototropism in response to blue light. They showed normal green sensitivity and normal second positive curvature leading to the hypothesis that two different photoreceptors mediate phototropism (Khurana and Poff, 1989). However, Liscum and Briggs (1995) demonstrated that null mutants at the *NPH1* locus, generated by neutron bombardment, were impaired in both first and second positive phototropic responses and in their response to green light. Furthermore, it was shown that these seedlings were also impaired in positive phototropism of the hypocotyl and negative phototropism of the roots. These studies suggest that the 124 kD plasma membrane associated protein, hypothesised to be the product of the *NPH1* gene, plays an important role in all of the phototropic responses investigated.

1.2.6.5 NPH1 is a Protein Kinase with a Putative Redox-Sensing Domain

Recently, Huala *et al.* (1997) cloned and sequenced the *NPH1* locus in *Arabidopsis*. They found that the gene encodes a protein of 996 amino acids and the coding region consists of 20 exons extending over a region of 5.4 kb. Analysis of the predicted amino acid sequence suggested that the carboxy-terminal region of NPH1 encodes a serine-threonine protein kinase. This NPH1 kinase domain contains the 11 sequence motifs typical of protein kinases and appears to be a member of the PVPK1 family of serine-threonine protein kinases (Hanks and Hunter, 1995). This is consistent with previous evidence which indicated that the 120 kD phosphoprotein from maize and pea is phosphorylated only on serine and threonine residues and is itself a kinase that mediates autophosphorylation.

Interestingly, hydrophobicity analysis of NPH1 suggests that it is a soluble protein without membrane-spanning domains. However, Raymond *et al.* (1992b) showed that

the phosphoprotein thought to be NPH1 is associated with the plasma membrane on isolation. The nature of its association with the plasma membrane remains to be seen.

Analysis of the amino-terminal portion of the predicted NPH1 protein sequence revealed two stretches of 107 amino acids which share 43% identity and 61% similarity. These repeats have been named LOV1 and LOV2 domains because of their similarity to the LOV motif found as a single copy in proteins from archeabacteria, eubacteria and eukaryotes. These proteins appear to have very diverse functions and otherwise share no similarity with NPH1. However, those proteins for which a function is known are regulated by environmental factors that could change their redox status: light, oxygen or voltage (and hence LOV).

It has been suggested that a possible function for the LOV domain is as a flavin binding site with the bound flavin acting as a redox sensor. Although at least one other protein is a flavoprotein (Hill *et al.*, 1996), we do not yet know whether FAD binds the protein within the LOV domain. If the LOV domain is indeed a flavin attachment site, it shares little or no sequence similarity with known flavin-binding sites such as those of the CRY1 and CRY2 photoreceptors. It would appear that elucidation of the role of the LOV domains, conserved during evolution, will prove very important in identifying the mechanism of action of NPH1.

1.2.6.6 CRY1 and CRY2 are Implicated in Phototropism

The cloning and sequencing of NPH1 in *Arabidopsis* confirmed that, as was previously thought, the NPH1 gene product is distinct from the UV/blue photoreceptors already identified. Indeed, several lines of evidence had previously suggested that the NPH1 protein is distinct from the CRY1 and CRY2 photoreceptors. For example, the molecular weight of NPH1 (124 kD) distinguishes it from CRY1 (75 kD) and CRY2 (approximately 68 kD based on the CRY2 gene encoding 608 amino acids). NPH1 has

been shown to be located at the plasma membrane whereas CRY1 is thought to be a soluble protein (Short *et al.*, 1992; Ahmad and Cashmore, 1993). Additionally, NPH1 lies on chromosome 3 (Huala *et al.*, 1997) and is therefore distinct from CRY2 which has been mapped to chromosome 1 (Ahmad *et al.*, 1998a). Genetic evidence has also indicated that NPH1 is distinct from CRY1 in that the *cry1 (hy4)* mutant exhibits normal phototropism and wild-type levels of the 124 kD protein (Liscum and Briggs, 1995). Similarly, *cry2 (fha1)* mutant seedlings showed unaltered first positive curvature (Ahmad *et al.*, 1998a).

Because of this evidence and the fact that genetic studies indicated that NPH1 appeared very early in the phototropism signal transduction pathway, NPH1 had often been referred to as a "putative photoreceptor for phototropism". Recently, however, Ahmad *et al.* (1998b) performed a study which suggests that CRY1 and CRY2 photoreceptors are involved in the phototropic response. Previous analysis using transgenic plants overexpressing CRY1 and CRY2 fusion constructs suggested considerable functional overlap between CRY1 and CRY2 (Ahmad *et al.*, 1998a) and therefore *cry1cry2* double mutants were produced in order to investigate this observation further.

Interestingly, the *cry1cry2* double mutant showed no evidence of first-positive phototropism. Furthermore, transgenic *Arabidopsis* seedlings overexpressing either CRY1 or CRY2 showed hypersensitivity to blue light during first-positive curvature. There was little difference in second positive curvature amongst the mutant and overexpressing lines tested which perhaps suggests the presence of blue light-absorbing species other than the cryptochromes involved in phototropism. However, the authors conclude that, given their effect on first-positive phototropism, the cryptochromes have an important role to play in the phototropic response.

The role of cryptochrome in the blue light-dependent autophosphorylation of NPH1 was also investigated in the *cry1cry2* double mutant. It was shown that levels of the

approximately 120 kD protein, thought to be NPH1, did not appear to be affected by the lack of cryptochrome. However, light-dependent phosphorylation of the 120 kD band was shown to be absent in *cry1cry2* seedlings, in contrast to wild-type, at the light intensities significant for phototropism.

These observations suggest that phosphorylation of the 120 kD protein, which is dependent on low-fluence blue light, seems to result from the action of cryptochrome (either CRY1 or CRY2). Given that NPH1 is a protein kinase with a putative redox-sensing domain (Huala *et al.*, 1997), then NPH1, if it is a flavoprotein, might serve as an acceptor for light-dependent electron transfer mediated by CRY1 or CRY2. Alternatively, NPH1 may be the primary photoreceptor for phototropism but CRY1 and CRY2 have important roles to play in modulating the response.

1.3 UV-B PERCEPTION

It is well documented that UV-B irradiation has a number of effects on plants, some of which are deleterious. UV-B light is an effective inhibitor of hypocotyl elongation and has also been shown to induce changes in cuticular wax composition, epidermal deformation, destruction of plasma membrane-associated ATPases and DNA damage (Tevini and Teramura, 1989; Stapleton, 1992). Plants have developed a number of mechanisms to protect themselves from the deleterious effects of UV-B irradiation. An example of this is the accumulation of flavonoids, which function as UV protectants, in response to harmful levels of UV light (Lois, 1994; Stapleton and Walbot, 1994). Flavonoids and anthocyanins generally accumulate in the epidermis thus protecting the underlying photosynthetic tissue from UV irradiation (Schmeltzer *et al.*, 1988; Hahlbrock and Scheel, 1989). Mutants defective in flavonoid biosynthesis are much

more sensitive to UV irradiation than wild-type plants (Li *et al.*, 1993; Lois and Buchanan, 1994).

Genes encoding the key enzymes in flavonoid biosynthesis are induced by UV radiation (Chappel and Hahlbrock, 1984; Hahlbrock and Scheel, 1989). One such gene encodes the enzyme chalcone synthase (*CHS*). Evidence suggests that *CHS* gene expression in response to UV-B light involves a separate photoreception system from phytochrome and CRY1. *CHS* gene expression was shown to be unaltered in response to UV-containing white light when PHYA and PHYB are absent (Batschauer *et al.*, 1996). Further, Fuglevand *et al.* (1996) reported that a *hy4* null mutant, lacking any detectable level of CRY1, retained normal induction of *CHS* transcripts in response to UV-B light. This evidence suggests that a separate photoreceptor system mediates UV-B responses. However, very little information is available regarding the nature of the putative UV-B photoreceptor(s).

It has been suggested that flavins or pterins are likely chromophores for UV-B photoreceptors (Gailand and Senger, 1988a, 1988b). This hypothesis is supported by studies described by Khare and Guruprasad (1993), who found that flavin antagonists inhibited the UV-B induction of anthocyanin formation in maize. Furthermore, riboflavin has been shown to enhance the induction of *CHS* gene expression in response to UV-B in parsley cells (Ensminger and Schäfer, 1992). Several studies have identified a number of mutants with altered responses to UV-B (Jenkins *et al.*, 1995). However, these mutants were altered in DNA repair or deficient in critical enzymes required for the synthesis of UV protecting flavonoids and provide no information on UV-B perception. It appears that production of mutants specifically altered in UV-B perception will be a vital step towards understanding this important facet of light regulation. It should also be noted that non-photoreceptor mediated UV-B perception may play a role in UV-B perception. UV-B irradiation induces changes in cells, for

example chemical changes in DNA and production of reactive oxygen species (ROS), these changes could act as the signal to induce a specific response (see 1.4.3).

1.4 SIGNAL TRANSDUCTION

1.4.1 Phytochrome Signal Transduction

Much of our understanding of phytochrome signal transduction has come from microinjection studies. A single cell system using the phytochrome-deficient *aurea* mutant of tomato has been devised to characterise components of the phytochrome signal transduction pathway (Neuhaus *et al.*, 1993). Microinjection of oat PHYA into single cells of the *aurea* mutant resulted in chloroplast development, anthocyanin accumulation and photosynthetic gene expression. This rescued phenotype was only seen in cells injected with PHYA and not in neighbouring cells, demonstrating that phytochrome signalling is cell autonomous. Although some debate exists as to whether all phytochromes or only PHYA is defective in this species (Barnes *et al.*, 1995), this experimental system provides an excellent means of identifying possible signalling steps downstream of phytochrome. Neuhaus *et al.* (1993) went on to show that G-protein activators, GTP- γ S and cholera toxin, induce wild-type chloroplast development and anthocyanin production in the *aurea* mutant. This observation suggests that G-protein activation is an early event in phytochrome signalling. These findings are consistent with previous reports indicating that heterotrimeric G-proteins are involved in the phytochrome-regulated signal transduction cascade (Bossen *et al.*, 1990; Romero *et al.*, 1991; Romero and Lam, 1993).

Further microinjection studies, using agonists and antagonists of classical signal transduction components, and pharmacological studies using a soybean cell culture have indicated that the light signal splits into two separate pathways after regulation by

heterotrimeric G-protein(s) (Neuhaus *et al.*, 1993; Barnes *et al.*, 1995; Bowler *et al.*, 1994a; Bowler *et al.*, 1994b). These pathways are the calcium/calmodulin-dependent and cGMP-dependent pathways which are coupled to the transcription of specific genes and chloroplast development. In addition, expression of some genes requires both signalling pathways. Interestingly, it was proposed that the cGMP-dependent and the calcium-dependent pathways showed reciprocal negative regulation. That is, high activity of the calcium-dependent pathway inhibits the cGMP-dependent pathway and *vice versa*. Bowler *et al.* (1994b) hypothesised that the physiological significance of this type of regulation may be to prevent the production of photosynthetic components (via both pathways) in the absence of photoprotective pigments which are produced by the cGMP-dependent pathway. It is perhaps worthy of consideration that although the microinjection technique has been very useful to study phytochrome signal transduction, it may not be suitable to identify novel signalling molecules which have not been identified in other systems.

Our present understanding of phytochrome signal transduction indicates that signal transduction mechanisms identified in animal systems also operate in plants. However, there are a few differences. For instance, cAMP is an important signalling molecule in many animal signalling mechanisms but appears to be much less significant in plants (Trewavas and Gilroy, 1991; Bolwell, 1995). In some cases, plant signalling has shown similarities to bacterial signal transduction (Hughes, 1994) whereas in others it may well be altogether novel (Deng *et al.*, 1992; Terzaghi and Cashmore, 1995b). Although Chua and co-workers in particular have made considerable advances, there are still many questions concerning phytochrome signalling that remain to be addressed. For example, phytochrome appears to be a cytosolic protein and yet mediates its effects via heterotrimeric G-proteins which are normally coupled to transmembrane receptors. Is there a requirement for an additional, perhaps novel, component to mediate this interaction? The calcium-dependent, cGMP-dependent and the calcium/cGMP-dependent pathways have been linked to PHYA and it will be very interesting to find

out whether these pathways are shared with other phytochromes or even UV/blue photoreceptors (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994a).

1.4.2 UV/Blue Light Signal Transduction

At present, our knowledge of UV-A/blue signal transduction is fragmentary and less advanced than that of phytochrome signalling. Progress has been made identifying likely signalling events associated with UV-A/blue photoreception. However, further work is required to relate these events to specific photoreceptors and particular downstream responses (Kaufman, 1993; Jenkins *et al.*, 1995).

1.4.2.1 UV/Blue Light-Induced Changes in Membrane Potential

Electrophysiological studies have been employed to identify UV-A/blue light-mediated effects on plant membranes. Spalding and Cosgrove (1989) demonstrated a large transient blue light-induced depolarisation of the plasma membrane in cucumber hypocotyls. This depolarisation occurred before the inhibition of hypocotyl elongation by blue light and it was suggested that the change in potential may reflect a signal transduction step associated with this response. Spalding and Cosgrove (1992) extended this study to investigate the effects of various ion channel inhibitors on this response. It was found that vanadate which is an inhibitor of the plasma membrane proton-ATPase, significantly reduced the depolarisation event. Further, KCN which is believed to block ATP production and thus inhibit the proton-ATPase, was also effective.

Given that the proton-ATPase is an important factor controlling the polarised state of the plant plasma membrane (Spanswick, 1981), it was hypothesised that blue light could mediate a membrane depolarisation by inhibiting proton-ATPase activity. It was also suggested that anion channels might be activated during this process since proton pump

inhibition would be insufficient to account for the full extent of the depolarisation. This hypothesis was initially supported by the observation that blue light also caused a rapid increase in cellular ATP levels. Recently, this hypothesis has been further supported by the observation that an anion channel is activated by blue light in *Arabidopsis* hypocotyls (Cho and Spalding, 1996). Similar blue light-induced depolarisation events have been observed in *Phaseolus* pulvinar motor cells (Nishizaki, 1988). This membrane potential change is thought to be associated with blue light-regulated leaf movement and may also involve inactivation of the proton-ATPase (Nishizaki, 1994).

In contrast, blue light has been implicated in the activation of plasma membrane proton-ATPase in stomatal guard cells. Proton extrusion is believed to play an important role in guard cell opening (Shimazaki *et al.*, 1986; Assmann *et al.*, 1985) and an involvement of the proton-ATPase in blue light-stimulated proton extrusion has been demonstrated by the inhibitory effects of vanadate (Gepstein *et al.*, 1987; Schwartz *et al.*, 1991; Amodeo *et al.*, 1992). In addition, an activator of the proton-ATPase (fusicoocin) has been shown to induce swelling of *Vicia* guard cell protoplasts (Assmann and Schwartz, 1992; Shimazaki *et al.*, 1993; Goh *et al.*, 1995). It should be noted, however, that Shimazaki *et al.* (1986) detected no inhibition of both proton extrusion and stomatal opening by vanadate in *Vicia* guard cells. Perhaps this discrepancy may be explained by problems in vanadate uptake (Schwartz *et al.*, 1991). These findings suggest that blue light regulates the activity of the plasma membrane proton-ATPase. However, the mechanism(s) involved is yet to be determined.

1.4.2.2 Redox Reactions and Electron Transport

Convincing evidence exists which suggests that redox activity and electron transport occur at the plasma membrane of both plant and animal cells (Rubinstein and Luster, 1993). Furthermore, it appears that there is redox activity at the plasma membrane in several blue light responses in a number of plant species (Jenkins *et al.*, 1995). In

particular, it has been postulated that a blue light-activated plasma membrane system, distinct from the proton-ATPase, is responsible for proton pumping by stomatal guard cells (Raghavendra, 1990). Vani and Raghavendra (1989) demonstrated a blue light-induced redox activity at the plasma membrane of *Vicia* stomatal guard cells which reduced tetrazolium salts. This activity had a requirement for NADH and was insensitive to the proton-ATPase inhibitor KCN. Similarly, redox activity identified in *Pisum* guard cell protoplasts is unaffected by vanadate and fusicoccin (Vani and Raghavendra, 1992). In both cases, the insensitivity to ATPase inhibitors or fusicoccin indicates that the guard cell redox activity does not involve the plasma membrane proton pump.

Further evidence for the presence of a distinct redox system is the observation that *Commelina* guard cell protoplasts incubated with the electron acceptor ferricyanide show reduced proton efflux in response to blue light (Gautier *et al.*, (1992). The authors noted that proton pumping was accompanied by oxygen uptake and suggested that oxygen might function as the terminal electron acceptor of the redox chain. However, it is suggested that this plasma membrane redox system is not a sufficient driving force of all the proton efflux required for K⁺ uptake during stomatal opening (Vani and Raghavendra, 1992). Thus further research is needed to establish the relative contributions of the redox system and the proton-ATPase to proton efflux.

Blue light-induced plasma membrane electron transport has also been reported in *Avena* mesophyll cells (Dharmawardhane *et al.*, 1989). It is interesting to note that the effect of blue light on electron transfer could only be detected in leaf segments pre-treated with inhibitors of protein kinase C. This suggests that de-phosphorylation of a redox protein(s) may be essential for the stimulation of electron transport in *Avena* mesophyll cells.

There is also evidence from cucumber which suggests that a plasma membrane redox activity is involved in the suppression of hypocotyl extension. The reducing agent, ascorbate, was found to prevent the inhibition of hypocotyl elongation in response to blue light (Shinkle and Jones, 1988). More recently, ascorbate and other reducing compounds, have been shown to enhance the blue light-induced phosphorylation of a 100 kD protein in plasma membranes isolated from maize coleoptiles (Hagar *et al.*, 1993). The properties of the maize protein are similar to the protein involved in phototropism around 120 kD in size identified in a number of plant species (Reymond *et al.*, 1992a; Liscum and Briggs, 1995). The reducing agents may therefore facilitate the transfer of electrons closely associated with blue light photoreception and/or protein phosphorylation. When taken together, these observations suggest that electron transfer is an important signalling event following UV-A/blue light perception. Given that CRY1 contains a flavin and a pterin chromophore (Lin *et al.*, 1995b; Malhotra *et al.*, 1995), it is possible that redox reactions and electron transport are early steps in CRY1 signal transduction.

1.4.2.3 G Proteins

It is well established that a range of GTP-binding proteins exist in higher plants (Terry *et al.*, 1993; Kaufman, 1994). It is also well documented that G protein activation is an early event in the phytochrome signalling pathways (Bossen *et al.*, 1990; Romero *et al.*, 1991; Neuhaus *et al.*, 1993). Separate studies have provided evidence that G proteins also play a role in blue light signal transduction. Warpeha *et al.* (1991) reported that blue light stimulates a transient increase of GTPase activity in plasma membrane-enriched fractions isolated from etiolated pea apices. Interestingly, only a very brief illumination (15 s) and a low fluence of blue light was required for this rapid response and it appeared that red light had no effect. Further, this blue light-mediated GTPase activity could be completely abolished by non-hydrolysable competitive inhibitors of heterotrimeric G-proteins, GTP γ S and imidodiphosphate (GppNHp).

Blue light did not induce [γ - 32 P] ATP hydrolysis in this assay, indicating the reaction was specific for GTP and blue light irradiation stimulated binding of the radiolabelled GTP analogue, GTP [γ - 35 S] to the pea plasma membrane fraction. This activity was assigned to a 40 kD plasma membrane protein which reacts with antibodies raised against mammalian G-protein α -subunits, including transducin- α . Furthermore this 40 kD polypeptide identified was ADP-ribosylated by pertussis and cholera toxins, a feature exhibited by particular mammalian G-proteins (Kaufman, 1994). It was also shown that blue light promoted binding of a GTP photoaffinity label to a polypeptide of similar size. This suggests that the 40 kD protein is an α -subunit of a typical heterotrimeric G-protein involved in a blue light signalling pathway of an unidentified low fluence response.

Homologues of genes encoding heterotrimeric G-protein subunits have been cloned from plants (Ma *et al.*, 1990; Ma *et al.*, 1991; Ishida *et al.*, 1993; Weiss *et al.*, 1994). However, none of these have been shown to be directly involved in light signalling. In addition, genes encoding homologues of small GTP-binding proteins, similar to the animal ras-related superfamily which are involved in various modes of signal transduction, have been cloned and the expression of some of these appears to be modulated by light (Nagano *et al.*, 1993; Nagano *et al.*, 1995; Yoshida *et al.*, 1993; Sommers and Song, 1994; Terry *et al.*, 1993).

1.4.2.4 Calcium

Calcium is known to function as an important second messenger in a variety of plant responses (Bush, 1993). However, the evidence for calcium involvement in UV-A/blue light signal transduction had been rather limited until recently. Amongst the earlier studies, for instance, is the observation by Russ *et al.* (1991) that UV-A and to a lesser extent blue light, stimulate an increase in cytosolic calcium in the green alga *Mougeotia*. The authors proposed that the increase in cytosolic calcium is involved in

light-regulated chloroplast movement. Other studies, often involving the use of calcium chelators and calcium channel blockers, provide indirect evidence that there is a requirement for calcium in UV-A/blue light signalling. For example, Shinkle and Jones (1988) demonstrated, using the calcium chelator EGTA, that the blue light inhibition of hypocotyl elongation in cucumber seedlings requires extracellular calcium. This response is likely to be specific to blue light, given that the cucumber seedlings were previously grown in red light.

More recently, however, Christie and Jenkins (1996) provided much more specific information on the role of calcium in UV/blue light signalling. Using a photomixotrophic *Arabidopsis* cell suspension culture, pharmacological experiments were performed which indicated that calcium was involved in both the UV-B and the UV-A/blue signal transduction pathways responsible for the control of chalcone synthase (*CHS*) gene expression. The induction of *CHS* transcripts in response to UV-B and UV-A/blue light was strongly inhibited by both nifedipine, which inhibits voltage-gated calcium channels, and ruthenium red, which inhibits calcium channels in vacuolar membranes and probably other membranes. However, this inhibition was not seen with lanthanum, which competes externally with calcium for uptake into the cells, nor with verapamil, a different calcium channel blocker. These observations suggest that specific types of calcium channel are involved.

The lanthanum and ruthenium red data together indicate that the UV-B and UV-A/blue light induction of *CHS* may require calcium flux across an internal membrane and therefore a specific pool of cellular calcium may be involved. However, attempts to induce *CHS* expression by artificially elevating cytosolic calcium levels with the ionophore A23187 and high concentrations of external calcium were unsuccessful. This was despite levels of cytosolic calcium being increased, demonstrated by induction of the *TCH3* (*TOUCH3*) gene which is regulated by cytosolic calcium. The authors propose that in order to produce *CHS* induction, the increase in cytosolic calcium must

be accompanied by another essential signal produced by UV-B or UV-A/blue illumination or, alternatively, that the increase in calcium must be localised, for example, in a specific microdomain at a particular intracellular membrane or in a specific organelle.

1.4.2.5 Calmodulin and Calcium-Dependent Kinases

Christie and Jenkins (1996) also demonstrated that calmodulin is involved in the transduction of UV-B light signals. The calmodulin antagonist, W-7, strongly inhibited the UV-B induction of *CHS* expression, but not the UV-A/blue light induction. This suggests that there are separate signal transduction pathways for UV-B induction and UV-A/blue induction of *CHS* expression. This observation is consistent with previous genetic evidence that the *hy4* null mutant retained normal induction of *CHS* expression in response to UV-B light but not to UV-A/blue light (Fuglevand *et al.*, 1996). Thus, in this case, it appears that different photoreceptors transduce signals along separate pathways to regulate *CHS* gene expression.

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

These results are in agreement with the observation that light-induced stomatal opening in *Commelina* epidermal strips is also inhibited by W-7 and ML-9. It appears, therefore, that a MLCK-like protein may be involved in the signal transduction pathway

coupling blue light photoreception to proton efflux in stomatal guard cells. Subsequently, it was demonstrated that fusicoccin restores proton pumping and stomatal opening previously inhibited by W-7 and ML-7 (Shimazaki *et al.*, 1993). Thus the inhibitory action of these compounds is specific to the light signalling pathway and not due to a direct effect on the proton pump.

1.4.2.6 Protein Kinases and Phosphatases

The role of protein kinases and phosphatases in light signalling is fairly well documented but not well understood. Perhaps the best studied example is that of the NPH1 protein in *Arabidopsis* which is believed to autophosphorylate in response to blue light and is involved in phototropic curvature (Huala *et al.*, 1997; Short and Briggs, 1994). Genetic evidence suggests that NPH1 mediates its effects very near the photoreceptor end of the signal transduction pathways (Liscum and Briggs, 1995; Ahmad *et al.*, 1998b).

In addition, an evacuated parsley protoplast system has been used to show that short-term irradiation with different fluences of red or white light results in rapid changes (within 20 s) in the phosphorylation pattern of several cytosolic proteins (Harter *et al.*, 1994). It should also be noted that protein phosphatases were recently shown to be required for light induction of chlorophyll accumulation and photosynthetic gene expression (Sheen, 1993). In etiolated maize seedlings, okadaic acid, a potent and specific inhibitor of protein phosphatase 1 and protein phosphatase 2A, completely prevented greening at 1 mM, whereas protein kinase inhibitors had no effect. Furthermore, in a maize protoplast transient assay system, okadaic acid had a concentration-dependent inhibition on two representative photosynthetic gene promoter-CAT' reporter constructs (Sheen, 1993). In addition, Christie and Jenkins (1996) demonstrated, using specific inhibitors, that serine/threonine protein kinase activity and protein phosphatase activity are required for the UV-B and UV-A/blue light induction of

CHS expression in *Arabidopsis* cell culture. However, specific targets of these kinase and phosphatase activities will have to be identified before their significance can be fully understood.

1.4.2.7 Inositol Phospholipid Metabolism

The inositol phospholipid pathway, an important aspect of animal signalling, is also operative in plants but has proved difficult to characterise (Trewavas and Gilroy, 1991). This pathway involves the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C in the plasma membrane to release the second messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). In animal systems, DAG activates protein kinase C whereas IP₃ releases calcium from intracellular stores thus activating calcium-dependent enzymes and altering cell physiology.

Although several components of the inositol phospholipid pathway are present in plant cells, evidence suggests that there are significant differences between the plant and animal phosphoinositide systems (Drøbak, 1992). An example of this is that no functional equivalent for protein kinase C appears to exist in higher plants. However, light-induced phospholipid turnover has been observed in *Samanea pulvini* (Morse *et al.*, 1987). It was demonstrated that only a brief irradiation of excised pulvini with white light (15-30 sec) is required to decrease PIP₂ levels and increase the levels of IP₃ and DAG. Although Morse *et al.* (1989) proposed that a blue light-absorbing pigment was involved in this light-stimulated reaction, no evidence for this has been published. Indeed, the light-stimulated phospholipid turnover may well be due to the activation of phytochrome, given that phytochrome has been shown to control phospholipid turnover in maize (Guron *et al.*, 1992).

1.4.3 UV-B Signal Transduction

At present, very little information is available regarding UV-B signal transduction in plants. Perhaps the best attempt to elucidate UV-B signal transduction has come from the study of UV/blue light regulation of *CHS* gene expression by Christie and Jenkins (1996) described above. They demonstrated the involvement of specific calcium channels and calmodulin in UV-B induction of *CHS* gene expression in an *Arabidopsis* cell culture.

More information on UV-B signal transduction has come from a study on the pathogenesis-related protein PR-1 from tobacco. Green and Fluhr (1995) reported that UV-B induces PR-1 accumulation in tobacco leaves and this was preceded by increased amounts of *PR-1* transcripts. Pre-treatment of tobacco leaves with cycloheximide inhibited *PR-1* transcript accumulation, indicating a requirement for cytoplasmic protein synthesis. In addition, the antioxidants *N*-acetyl-L-cysteine and pyrrolidine dithiocarbamate inhibited UV-B-induced PR-1 accumulation whereas rose bengal, a generator of active oxygen, caused an accumulation of PR-1 in the absence of UV-B. The authors therefore proposed that the signalling pathway coupling UV-B perception to PR-1 accumulation involves protein synthesis and the generation of reactive oxygen species (ROS). Interestingly, UV-C irradiation has been shown to increase levels of ROS in rose cells (Murphy and Huerta, 1990). Furthermore, UV-induced defence responses in animal cells have also been reported to involve ROS (Devary *et al.*, 1992). Perhaps similar defence mechanisms in response to UV light may exist in plant and animal systems.

1.5 SIGNAL TRANSDUCTION MUTANTS

1.5.1 Genetic Analysis of Light Signal Transduction

Genetic screens have proved fruitful in identifying some of the regulatory components involved in light-regulated seedling development. Many of these photomorphogenic mutants appear to affect signal transduction events subsequent to photoreception. Based on the contrasting morphology of light- and dark-grown seedlings, these screens have produced two types of loss-of-function mutants. Mutations in positive regulators of photomorphogenesis result in a *hy* (elongated hypocotyl) phenotype which is a partial etiolated phenotype of seedlings grown under defined light conditions. The *hy* mutants include photoreceptor mutants (for instance *hy4*) as well as downstream signalling components such as *hy5*, *fhy1* and *fhy3* (Ahmad and Cashmore, 1993; Koornneef *et al.*, 1980; Whitelam *et al.*, 1993). In contrast, mutations in negative regulators result in a *cop* (constitutive photomorphogenesis) or *det* (de-etiolated) phenotype whereby dark-grown seedlings aberrantly undergo photomorphogenesis (Deng, 1994; Chory, 1993).

1.5.2 Positive Effectors

Amongst the group of long hypocotyl mutants which display the dark-grown characteristic of long hypocotyls when germinated in the light are *fhy1* and *fhy3* (Whitelam *et al.*, 1993). These mutants show the long hypocotyl phenotype in far-red but not red, blue or white light. Their far-red insensitivity suggests that they are specifically involved in PHYA signalling. However, it has been demonstrated that neither are photoreceptor mutants because they both have wild-type levels of functional PHYA (Whitelam *et al.*, 1993). The mode of action and possible interactions between these loci remains unclear. Their molecular cloning will be an important step in the further study of these loci.

More information is available on the *hy5* mutant of *Arabidopsis*. This mutant was isolated in a white light screen but shows the long hypocotyl phenotype in far-red and red light and to a lesser extent in blue and UV light (Koornneef *et al.*, 1980; Ang and Deng, 1994). These initial results suggest that this locus may be involved in a downstream signalling cascade after the convergence of signals from multiple photoreceptors. Subsequent studies have revealed that this hypothesis may well be correct. The *HY5* gene has been cloned and shown to encode a 168 amino acid protein representing a member of a new class of basic leucine zipper (bZIP) DNA binding proteins (Oyama *et al.*, 1997). A number of bZIP proteins have been isolated from plants by their biochemical affinity to DNA sequences which have an ACGT core motif (Foster *et al.*, 1994; Menkens *et al.*, 1995). Further, Chattopadhyay *et al.* (1998) reported that HY5 is constitutively nuclear localised and specifically binds the G-box DNA sequence element (a well characterised light-responsive element) *in vitro*. It was also demonstrated that light activation of both synthetic and native promoters which contain G-box sequences was significantly compromised in *hy5* mutant plants. These results suggest that HY5 may interact directly with the G-box in the promoters of light-inducible genes to mediate light-controlled transcriptional activity.

1.5.3 Negative Regulators

The *cop/det*-type mutants can be classified into three general groups (see Figure 1.1). The first group of mutants exhibits a pleiotropic phenotype which appears to be light independent. That is, dark-grown mutant seedlings resemble their light-grown siblings in the pattern of light-regulated gene expression, in cell and plastid differentiation and in overall morphology. Mutants in this group have been identified at several *Arabidopsis* loci including *cop1*, *det1* and *cop8-15* (Chory *et al.*, 1989a; Deng *et al.*, 1991; Wei and Deng, 1992; Wei *et al.*, 1994a; Miséra *et al.*, 1994; Kwok *et al.*, 1996). These mutants will be the focus of this section because they are amongst the best characterised and most relevant in the study of light signal transduction.

Figure 1.1 Summary of photomorphogenic mutants in *Arabidopsis*

The photomorphogenic mutants of *Arabidopsis* are summarised in the following table, reproduced from Deng, 1994.

COP/DET Gene	Other Names	Dark-Grown Mutant Seedling Morphology	Lethality of Null Mutant	References
COP1	FUS1, EMB168	Pleiotropic	Yes	Deng et al., 1991
COP8	FUS8, EMB134	Pleiotropic	Yes	Wei et al., 1994
COP9	FUS7, EMB143	Pleiotropic	Yes	Wei and Deng, 1994
COP10	FUS9, EMB144	Pleiotropic	Yes	Wei et al., 1994
COP11	FUS6, EMB73	Pleiotropic	Yes	Wei et al., 1994; Castle and Meinke, 1994
COP12	FUS12	Pleiotropic	Yes	Miséra et al., 1994; Kwok et al., 1996
COP13	FUS11	Pleiotropic	Yes	Miséra et al., 1994; Kwok et al., 1996
COP14	FUS4	Pleiotropic	Yes	Miséra et al., 1994; Kwok et al., 1996
COP15	FUS3	Pleiotropic	Yes	Miséra et al., 1994; Kwok et al., 1996
DET1	FUS2	Pleiotropic	Yes	Chory et al., 1989
COP2	AMP1, PT1	Open cotyledons	No	Hou et al., 1993; Lehman et al., 1996; Chaudhury et al., 1993
COP3	HLS1	Open cotyledons	No	Hou et al., 1993; Lehman et al., 1996
COP4	COP7	Open cotyledons	Unknown	Hou et al., 1993; Lehman et al., 1996
DET2		Short hypocotyl	No	Chory et al., 1991
DET3		Short hypocotyl	Unknown	Cabrera y Poch et al., 1993
PRC1		Short hypocotyl	Unknown	Desnos et al., 1996
DIM		Short hypocotyl	Unknown	Takahashi et al., 1995
CPD		Short hypocotyl	No	Szekeres et al., 1996

The second group of mutants have opened cotyledons without apical hooks but have elongated hypocotyls and show normal etioplast development when grown in the dark. This group includes *cop2/ump1*, *cop3/hls1* and *cop4* (Chaudhury *et al.*, 1993; Hou *et al.*, 1993; Lehman *et al.*, 1996). Of these mutants, only *cop4* shows altered expression of light-regulated genes. When grown in the dark, *cop4* shows a moderate depression of nuclear-encoded photosynthetic genes (*CAB1* and the chlorophyll a/b binding protein genes) whereas *cop2* and *cop3* appear unaltered in light regulated gene expression (Hou *et al.*, 1993). The third group of mutants exhibit a short phenotype when grown in darkness for less than five days. It includes *det2*, *det3*, *dim*, *prc1* and *cpd* (Chory *et al.*, 1991; Cabrera y Poch *et al.*, 1993; Takahashi *et al.*, 1995; Desnos *et al.*, 1996; Szekeres *et al.*, 1996). Most of these mutants (except *prc1*) also exhibited significant cotyledon expansion and opening after growing for over a week in darkness. However, no sign of chloroplast development was observed.

The second and third groups of mutants exhibit partial light-dependent seedling development in darkness in contrast to the first group. Given that many factors other than light also affect seedling morphogenesis, for example metabolites, nutrients and hormones, it seems likely that some loci from the second and third groups will define components in these other signalling pathways. Indeed, this has already been demonstrated in several cases. For instance, Lehman *et al.* (1996) has shown that *cop3* is allelic to *hookless1* (*hls1*) which was isolated based on altered ethylene responsiveness. Furthermore, *cop4* has been shown to lack a normal gravitropic response and thus may also play a role in gravitropism (Chaudhury *et al.*, 1993). It therefore appears that some of these less pleiotropic loci may represent components of the “cross-talk” between the light regulatory network and other signalling pathways.

1.5.4 *Arabidopsis fusca* Mutants

A phenotype which is common to all severe alleles of the pleiotropic *cop/det* mutants is the accumulation of anthocyanins in the mature seed and young seedlings giving them a purple appearance. The *fusca* (from the latin 'dark purple') mutants of *Arabidopsis* were identified by screening for increased levels of these purple pigments. They are characterised by the synthesis of high levels of anthocyanin in immature embryos and seedlings and by adult lethality (Castle and Meinke, 1994; Miséra *et al.*, 1994). However, adult lethality is not due to the overproduction of anthocyanin as double mutants produced between *fusca* alleles and mutants defective in anthocyanin biosynthesis still exhibit abnormal development in the absence of anthocyanin (Castle and Meinke, 1994; Miséra *et al.*, 1994). Detailed characterisation of the twelve available *fusca* loci suggested that ten of them show pleiotropic photomorphogenic development in the dark (Miséra *et al.*, 1994; Kwok *et al.*, 1996). Furthermore, six of these are allelic to the pleiotropic *COP/DET* loci (*COP1*, *DET1*, *COP8-11*, see Figure 1) and four defined novel loci (Kwok *et al.*, 1996). Thus, a total of ten pleiotropic *COP/DET/FUS* loci have been identified.

As previously mentioned, these pleiotropic mutants share all of the characteristics of light-grown seedlings, including cell differentiation patterns, plastid differentiation and gene expression when grown in darkness, with the exception of chlorophyll accumulation (Chory *et al.*, 1989; Deng *et al.*, 1991; Wei and Deng, 1992; Wei *et al.*, 1994). Genetic analysis indicates that these loci act downstream of both CRY1 and phytochrome (Wei *et al.*, 1994; Kwok *et al.*, 1996; Chory, 1992; Ang and Deng, 1994; Wei *et al.*, 1994). Their pleiotropic mutant phenotypes imply that the wild-type gene products are involved in steps of the light signalling process before the branchpoint to photomorphogenic responses. In addition, their recessive nature suggests that the gene products normally act as negative regulators of photomorphogenic development in the dark and that light abolishes their activity.

1.5.5 Molecular Analysis of the Photomorphogenic Repressors

To date, four of the *COP/DET/FUS* loci have been cloned. These are *COP1*, *COP9*, *COP11/FUS6* and *DET1* (Deng *et al.*, 1992; McNellis *et al.*, 1994a; Wei *et al.*, 1994; Castle and Meinke, 1994; Pepper *et al.*, 1994). Interestingly, it appears that *COP9*, *COP11* and *DET1* have no sequence homology with other proteins. In contrast, the *COP1* locus encodes a 74 kD protein which contains a number of recognisable structural motifs including a zinc-finger domain, a putative coiled-coil region and a domain with multiple WD-40 repeats homologous to the β -subunit of heterotrimeric G proteins (Deng, 1992). Unfortunately, specific biochemical functions have not yet been established for these proteins. However, recent studies have provided some information on these proteins, especially *COP1*, which overexpression studies suggest is an autonomous repressor of photomorphogenesis. McNellis *et al.* (1994b) reported that overexpression of *COP1* in transgenic *Arabidopsis* seedlings leads to the partial suppression of photomorphogenesis. This was characterised by significant hypocotyl elongation under far-red and blue light conditions as well as photoperiodic white light, thus providing direct evidence which supports the hypothesis that *COP1* is a repressor of photomorphogenesis.

1.5.6 *COP1* Localisation is Regulated by Light

Studies investigating the cellular localisation of *COP1*, when fused to a GUS reporter, have revealed a possible mechanism by which *COP1* activity is regulated by light. It was demonstrated that the GUS-*COP1* fusion protein is specifically enriched in the nucleus in the dark and is excluded from the nucleus under light conditions (von Arnim and Deng, 1994). Initially, this observation suggested that white light may inactivate *COP1* repression by specific nuclear depletion. It is worthy of note that the GUS-*COP1* fusion protein used in this study was biologically active as demonstrated by the observation that it, when overexpressed in transgenic plants, produced a long hypocotyl

phenotype (von Arnim and Deng, 1994). However, light-mediated migration of GUS-COP1 out of the nucleus took almost 12 hours to be detected and reached a maximum after 36 hours (von Arnim and Deng, 1994). Given that many light-regulated developmental changes, for instance the inhibition of hypocotyl elongation, occur within minutes, these observations make it difficult to envisage COP1 functioning as a direct repressor of photomorphogenesis. However, we must consider that the GUS-COP1 fusion is a larger protein than the endogenous COP1 protein and will probably have slower kinetics than the native COP1 protein. Nevertheless, it seems unlikely that nuclear exclusion in the light is the primary effector of the inactivation of COP1 repression. It seems more likely that nuclear exclusion serves as a mechanism to maintain, rather than initiate, the committed developmental program

1.5.7 Domains of the COP1 Protein

As previously mentioned, the COP1 protein has three recognisable structural domains (von Arnim and Deng, 1993). The N-terminus has a ring-finger-type zinc-binding domain that shares similarity with over 30 regulatory proteins, including several which are involved in transcriptional regulation, or that interact with DNA. The C-terminal half of COP1 contains a domain similar to the β -subunit of heterotrimeric G-proteins. This domain consists of multiple WD-40 repeats which are often present in proteins implicated in gene repression and signal transduction. A putative coiled-coil domain separates the zinc-finger domain from the WD-40 repeats.

The structure of COP1 suggests that it will have a role in the nucleus, perhaps as a regulator of transcription. This is supported by COP1 sharing homology in the putative coiled-coil region and WD-40 repeats with d-TAF₁₁₈₀ from *Drosophila* which is a component of the RNA polymerase II transcriptional apparatus (Dymlacht *et al.*, 1993). COP1 also shares homology in the WD-40 repeat region with a yeast protein,

designated TUP1, which is thought to be a global transcriptional repressor (Kingston *et al.*, 1996).

Interestingly, sequence analysis of several *cop1* mutant alleles indicated that disruption of the WD-40 repeat region of the extreme carboxy-terminal end of the protein results in a lethal allele (McNellis *et al.*, 1994a). Further, a mutant containing just the amino-terminal 282 amino acids of COP1 (N282) had only a weak phenotype which suggests that this region alone retains some activity. McNellis *et al.* (1996) performed structure-function studies of COP1, to further analyse its role. Transgenic *Arabidopsis* plants overexpressing the N282 fragment of COP1 showed hypersensitivity in the inhibition of hypocotyl elongation in light and a partial constitutive photomorphogenic phenotype in the dark. This suggests that the N282 fragment is capable of interacting with endogenous COP1 itself and/or with other downstream regulatory components. Indeed, McNellis *et al.* (1996) also demonstrated that the N282 fragment self-interacts in the yeast two-hybrid system.

A biochemical screen for proteins which interact has identified a protein designated CIP1 (Matsui *et al.*, 1995). This is a putative cytoskeletal related protein which may play a role in trafficking COP1 in and/or out of the nucleus. Interestingly, the interactive site on COP1 was found to be its coiled-coil region. However, further studies are required to identify the relative roles of these proteins *in vivo*.

1.5.8 COP9 and COP11 Act Within a Protein Complex

Recently, biochemical studies have revealed that the COP9 protein exists exclusively as part of a large protein complex. COP9 is a 23 kD protein and forms part of a complex thought to be around 560 kD in size and whose size and conformation may be modulated by light (Wei *et al.*, 1994). Interestingly, the steady state level of COP9 was significantly reduced in *cop8* and *cop11* mutants (Wei *et al.*, 1994). This observation

suggests that COP8 and COP11 are important either for the stability of the COP9 complex or for its formation. Recently, immunological studies have produced some interesting information on both COP9 and COP11. Using specific antibodies raised against the cloned *Arabidopsis* COP9 and COP11, it was demonstrated that the accumulation of COP9 and COP11 is co-ordinated throughout development in wild-type plants as well as in the pleiotropic *cop/det/fus* mutant backgrounds (Staub *et al.*, 1996). Both COP9 and COP11 co-fractionate into an identical high molecular weight complex in an analytical gel-filtration assay and neither protein was found in its monomeric form. Furthermore, both proteins were selectively co-immunoprecipitated from total cellular extracts by antibodies to either COP9 or COP11. These observations, when taken together, strongly suggest that both COP9 and COP11 are components of the COP9 complex in *Arabidopsis* (Staub *et al.*, 1996). There is additional evidence which supports this hypothesis. Purification of the complex from cauliflower heads has suggested that the complex is multisubunit in nature and comprises of COP9, COP11 and several novel proteins (Chamowitz *et al.*, 1996). These studies also revealed that the COP9 complex, in contrast to COP1, is nuclear localised and its localisation is not affected by light conditions or tissue types in *Arabidopsis* (Staub *et al.*, 1996; Chamowitz *et al.*, 1996).

1.5.9 Interactions Between Photomorphogenic Loci

Isolation of the *det/cop/fus* mutants and subsequent cloning of some of these loci have provided important insights into the complex signalling network associated with photomorphogenic development. We know that photomorphogenesis requires the concerted action of multiple photoreceptors and it seems likely that their signal transduction pathways converge downstream upon common regulatory elements. The pleiotropic phenotypes caused by the *det/cop/fus* mutations imply that these loci encode such common regulatory elements. Indeed, the results described above, together with the information that DET1 is also a nuclear localised protein although not a member of

the COP9 complex, suggest that all four cloned *COP/DET/FUS* genes (COP1, COP9, COP11 and DET1) encode nuclear regulators (Pepper *et al.*, 1994; Chamowitz *et al.*, 1996).

This raises the question as to what are the relationships between, COP1, DET1, the COP9 complex and the proteins encoded by other uncloned loci with the same mutant phenotypes. Studies performed using the GUS-COP1 fusion protein have provided some insights in this area. These studies have suggested that nuclear localisation of COP1 is defective in *cop8*, *cop9* and *cop11* mutants (Chamowitz *et al.*, 1996). It is reasonable, then, to suggest that the COP9 complex is directly involved in the nuclear translocation of COP1. This may be as components of a nuclear pore complex or as a more specialised transportation mechanism specific to COP1. It should be noted that nuclear import and retention of a control nuclear-targeted GUS-N1a fusion protein was not affected in these *cop* mutants (Chamowitz *et al.*, 1996). Further study is required to find out whether only nuclear localisation of COP1 is affected in these mutants or, interestingly, whether nuclear localisation of DET1 is also affected.

Genetic evidence suggests that COP1 acts within the same signalling pathway as both DET1 and HY5 (Ang and Deng, 1994). Double mutants between weak alleles of *cop1* and *det1* enhance each others phenotypes and cause synthetic lethality. It was also observed that the *hy5* mutation could suppress several phenotypes of a weak *cop1* allele but not of a null allele. These allele specific interactions between *hy5* and *cop1* suggest an interaction of their gene products (Ang and Deng, 1994). This prospect is an intriguing one given that HY5 is a positive effector of photomorphogenesis and COP1 is a negative effector of photomorphogenesis. One hypothesis is that COP1 acts to sequester HY5 in the dark, whereas light releases this association and allows HY5 to activate photomorphogenic development (Staub and Deng, 1996).

The *det3* mutant and to some extent *cop2* and *cop3* mutants have a de-etiolated morphology in the dark but exhibit a wild-type pattern of light-regulated gene

expression (Cabrera y Poch *et al.*, 1993; Hou *et al.*, 1993). This suggests that DET3, COP2, and COP3 are possible regulatory components defining downstream branches in the light-regulated signal transduction pathway. Similarly, Li *et al.* (1994) have isolated several *Arabidopsis* mutants which are specifically altered in one particular aspect of light-development. These so called *doc* mutants (*dark overexpression of CAB*) have normal etiolated morphology in the dark but exhibit aberrant *CAB* gene expression. Epistasis studies indicate that these new genes define downstream branches of the light signal transduction pathway that are specifically concerned with the regulation of *CAB* gene expression. Further genetic analysis is beginning to establish models outlining the regulatory hierarchy between photoreceptors and the different downstream signalling components (Ang and Deng, 1994). Genetic dissection of the light-signalling network is well underway and it will be important to couple this to biochemical analysis to define the signalling components encoded by the *COP/DET/FUS* loci.

1.5.10 *Arabidopsis icx1* mutant

Another interesting class of mutants are those which appear to have an enhanced sensitivity to light. An example of this is the high pigment mutant of tomato (*hp*) which shows an increased phytochrome-induction of anthocyanin formation in the absence of blue light (Peterson *et al.*, 1989). It is proposed that the *hp* mutant may be altered in a signalling component linking phytochrome and blue light signal transduction. More recently, Jackson *et al.* (1995) identified a mutant which is altered in anthocyanin formation and *CHS* expression. This mutant, designated *icx1* (*increased chalcone synthase expression*), was isolated by screening mutagenised transgenic seedlings for altered expression of GUS fused to the promoter of the *Sinapis alba* (white mustard) *CHS-1* gene. The *icx1* mutant has elevated GUS activity over a range of fluence rates of white light compared to the transgenic wild-type. Increased GUS activity was found to correspond with an increase in *CHS* transcript levels. Moreover, *CHI* and *DFR* transcript levels were also elevated as was anthocyanin production whereas the

phenotype of dark-grown *icx1* seedlings appeared to be normal. Therefore, the *icx1* mutant appears to have increased sensitivity to light with respect to light-regulated expression of genes involved in flavonoid biosynthesis. An interpretation of this recessive mutation is that *ICX1* encodes a negative regulator which normally constrains the expression of *CHS* and other genes in the light. Interestingly, no alteration in *CAB* transcript levels were observed, indicating that *icx1* is not altered in the expression of all light-regulated genes. The *icx1* mutant is also altered in several aspects of epidermal development, including a reduced level of leaf trichomes and alterations to the seed coat. Indeed, *icx1* is altered in similar aspects of the phenotype to the *ttg* (*transparent testa, glabra*) mutant which is deficient in trichomes, seed mucilage and anthocyanin synthesis (Koornneef *et al.*, 1981). As a consequence the seed testa of *ttg* is unpigmented. On the basis of these results it is likely that *ICX1* acts in the epidermis to control gene expression and development.

1.6 CHALCONE SYNTHASE GENE EXPRESSION

1.6.1 Coupling Signal Transduction to Gene Expression

Many photomorphogenic responses require changes in both nuclear and chloroplast gene expression. Therefore, in most cases, the binding of transcription factors to *cis*-acting regulatory sequences of promoters is considered to be the terminal step of a given light signal transduction pathway. Several genes have been used to define DNA-binding factors and *cis*-acting promoter elements controlling light-regulated transcription (Jenkins, 1991; Terzaghi and Cashmore, 1995a). One of the best studied systems in this area is the chalcone synthase (*CHS*) gene in parsley (Weisshaar *et al.*, 1991b).

Chalcone synthase catalyses the first committed enzymatic step in the flavonoid specific branch of the phenylpropanoid pathway (Hahlbrock and Grisebach, 1979). In many

plant species a variety of environmental, developmental and metabolic cues have been shown to be involved in the regulation of *CHS* expression. For instance, *CHS* expression is strongly induced during floral development and by light in *Petunia* or by elicitor and light in French bean and soybean (van Tunen and Mol, 1989). In cultured parsley cells, *CHS* expression is UV and blue light dependent although red light, diurnal rhythm and developmental state of the tissue have additional modulating effects (Kreuzaler *et al.*, 1983; Ohl *et al.*, 1989).

Early studies demonstrated that photocontrol of *CHS* expression in parsley cell suspension cultures is primarily regulated by UV/blue light and thus provided a convenient system in which to study the *CHS* promoter (Ohl *et al.*, 1989; Frohnmeyer *et al.*, 1992). Using an *in vivo* footprinting technique, Schulze-Lefert *et al.* (1989a) detected the appearance of four footprints in the *CHS* promoter of UV-treated cells. These four regions, absent in dark-treated cells and designated boxes I, II, III and IV, were considered to be potential sites for DNA-protein interactions. Subsequent transient expression studies in parsley cell culture protoplasts confirmed that the boxes defined by *in vivo* footprinting were important for promoter function. Deletion analysis of the *CHS* promoter revealed that boxes I and II form a light regulatory unit (LRU1) which is sufficient for light-regulated expression (Schulze-Lefert *et al.* 1989b; Weisshaar *et al.*, 1991a). Upstream boxes III and IV form a second light regulatory unit (LRU2) which can mediate light-induced expression in the absence of LRU1, although Unit 1 is more effective (Schulze-Lefert *et al.* 1989b). The authors suggested that LRU2 is a weak *cis*-acting element which can enhance the activity of LRU1. The presence of a region upstream from LRU2 was also found to enhance the light responsiveness of both units (Schulze-Lefert *et al.* 1989b). Both box I and box II are required for light-responsiveness as mutations of specific nucleotides within these elements leads to a loss of function (Block *et al.*, 1990).

It was observed that box II belongs to the G-box family of *cis*-acting elements, which is part of the larger family of ACGT-containing elements. G-box elements were first thought to be unique to light-induced genes but have been subsequently shown to reside in promoters of many other genes which are not regulated by light (Menkens *et al.*, 1995). Analysis of the box I sequence has shown it to be a member of the family of MYB recognition elements (Feldbrügge *et al.*, 1997). It is generally accepted that ACGT-containing elements and MYB recognition elements are bound by bZIP and MYB classes of transcription factors respectively (Foster *et al.*, 1994; Martin and Paz-Ares, 1997).

Studies have also shown that the *CHS-1* promoter of *Sinapis* mediates UV light-induced transient expression in the parsley transient expression system (Frohnmeyer *et al.*, 1992). A sequence with high homology to LRU1 in parsley has been identified in the promoter of the mustard *CHS* gene. Deletions in this region result in a loss of light-induced expression, indicating that similar elements control the UV-induced transcription of *CHS* genes in parsley and mustard (Rocholl *et al.*, 1994). More recently, studies have demonstrated that the *Sinapis* LRU1 is required for photocontrol and tissue-specific expression in transgenic *Arabidopsis* and tobacco seedlings (Kaiser *et al.*, 1995; Kaiser and Batschauer, 1995). Furthermore, the LRU1 from *Sinapis* is sufficient for transcriptional activation in response to a range of different light qualities (Rocholl *et al.*, 1994; Kaiser *et al.*, 1995). Similar promoter regions have been implicated in *CHS* promoter function in other species such as bean (*Phaseolus vulgaris*) (Faktor *et al.*, 1997).

1.6.2 Control of *CHS* Gene Expression in *Arabidopsis*

Although parsley, in particular, has proved to be an excellent system for defining the basic aspects of the control of *CHS* promoter activity, it is not an ideal system for the further dissection of the cellular and molecular mechanisms involved in the light

regulation of *CHS* gene expression because it is not well suited to a genetic approach. The availability of mutants would enable the functions of specific members of the families of transcription factors that interact with the *CHS* promoter to be defined. In particular, the contribution of the complex transcription factor families defined by bZIP and MYB-like DNA-binding domains needs to be analysed by genetic means (Jenkins, 1997). These transcription factor families are, according to biochemical data, involved in regulating light-responsive *CHS* promoter activity in parsley, and are likely to do so in *Arabidopsis* (Feldbrügge *et al.*, 1997; Foster *et al.*, 1994; Martin and Paz-Ares, 1997). It, therefore, seems reasonable to focus research in this area on *Arabidopsis*.

In *Arabidopsis*, *CHS* is encoded by a single gene whose expression is tightly regulated at the level of transcription (Feinbaum and Ausubel, 1988). *CHS* expression is regulated predominantly by light in the UV-B, UV-A and blue regions of the spectrum, and phytochrome regulation is confined to very young seedlings (Batschauer *et al.*, 1996; Feinbaum and Ausubel, 1988; Jackson *et al.*, 1995; Kaiser and Batschauer, 1995). It was shown, using a *hy4* null mutant (*hy4-2.23N*), that distinct photoreception systems mediate the inductive effects of UV-B and UV-A/blue light on *CHS* gene expression in *Arabidopsis* (Fuglevand *et al.*, 1996). These results indicated that the primary photoreceptor mediating the UV-A/blue response was CRY1 and whereas the UV-B response was mediated by an, as yet, unidentified photoreceptor. In addition, distinct phototransduction pathways act synergistically to maximise the stimulation of *CHS* expression (Fuglevand *et al.*, 1996). Christie and Jenkins (1996) have obtained initial information on the cellular signal transduction events that couple CRY1 and the UV-B photoreception system to *CHS* expression in *Arabidopsis*. Furthermore, mutants with a hyper-response to UV and blue light indicate that negative regulators are involved in the effector pathways for these responses (Jackson *et al.*, 1995; Jenkins, 1997).

Feinbaum *et al.* (1991) reported an initial study of the promoter elements of the *Arabidopsis CHS* gene (*AtCHS*) involved in light regulation. The structure of the *AtCHS* promoter is shown in comparison with *CHS* promoters from other species in Figure 1.2.

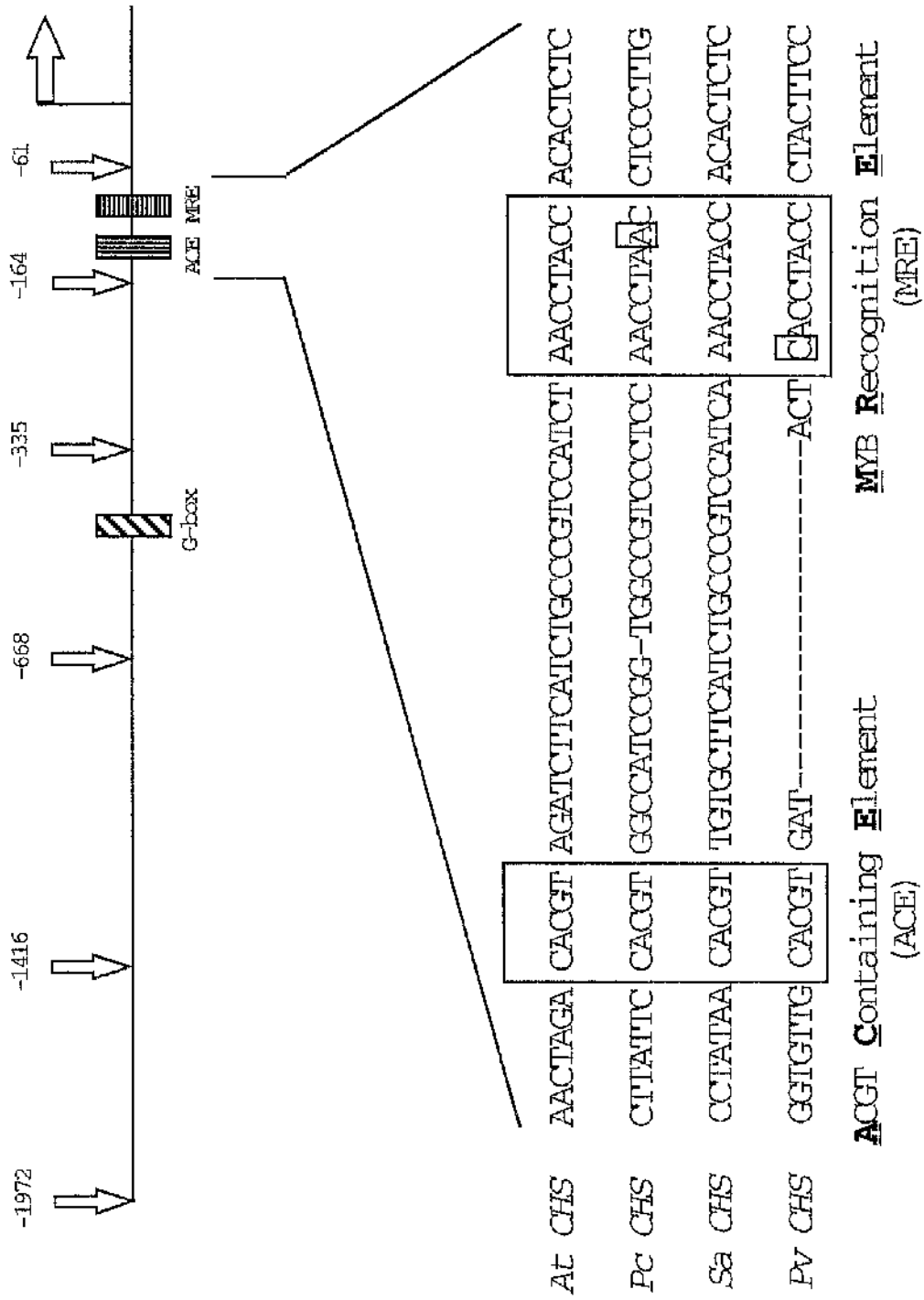
The *AtCHS* promoter contains a region, with an ACGT-containing element and a MYB recognition element, with striking similarity to the LRU1 sequence in the parsley promoter. In addition, a G-box-like sequence described by Feinbaum *et al.* (1991) is upstream of this region. This initial research has raised many questions regarding the *AtCHS* promoter, in particular the role of the region similar to the parsley LRU1 whose function was defined by Feinbaum *et al.* (1991). Study of the sequence elements of this promoter may well provide useful information regarding the terminal events of the UV-A/blue and UV-B light signal transduction pathways which drive expression of the *CHS* gene.

1.7 TRANSCRIPTION FACTORS INVOLVED IN THE REGULATION OF *CHS*

Research into the transcription factors which bind to the *Arabidopsis CHS* promoter in order to modulate its activity is still at an early stage. However, given the nature of the DNA sequence elements present in this promoter, it appears that members of the bZIP and MYB families of plant transcription factors are the best candidates. Several studies in other species, in particular parsley and maize, have provided some insight into the nature of these transcription factors and their interactions with light regulated promoters.

Figure 1.2 Structure of the *Arabidopsis CHS* promoter

The relative positions of conserved sequence elements as well as 5' termini of the promoter deletion variants are shown. Horizontally and vertically striped boxes labelled MRE and ACE indicate promoter regions related to MRE^{PcCHS} and ACE^{PcCHS}, respectively. The diagonally striped box represents the G-box-like sequence described by Feinbaum *et al.* (1991). Arrowheads mark the 5' termini of the promoter deletion variants and indicate the distance to the transcription initiation site. Sequence comparison of *Arabidopsis thaliana* (*At*), *Petroselinum crispum* (*Pc*), *Sinapis alba* (*Sa*) and *Phaseolus vulgaris* (*Pv*) *CHS* promoters are shown and the nucleotide positions conserved between the various species within the ACGT-containing element (ACE) and the MYB-recognition element (MRE) are highlighted in boxes.



1.7.1 Plant bZIP Transcription Factors

In a study of the regulation of *CHS* gene expression in parsley in response to UV-containing white light, Southwestern screening revealed three parsley cDNAs encoding proteins which specifically bind to the ACGT-containing element from the parsley LRU1 (Weisshaar *et al.*, 1991b). The deduced amino acid sequences of all three ACGT-binding proteins revealed conserved basic and leucine-zipper domains characteristic of bZIP-type DNA binding proteins. These parsley bZIP factors were designated *Common Plant Regulatory Factors* (CPRFs).

More than 30 plant bZIP proteins have been identified, most of them either by screening expression libraries with DNA ligand probes or by low stringency hybridisation with probes based on the conserved bZIP region (Foster *et al.*, 1994). All plant bZIP proteins so far characterised recognise ACGT-containing elements. This family is characterised by a basic region and a leucine zipper domain known as bZIP. This is a compact structure with a basic region which binds DNA in a sequence specific manner and the leucine zipper acts as a dimerisation interface. Dimerisation is a prerequisite (whether as a homo- or heterodimer) for DNA binding of bZIP proteins. The affinity with which a given plant bZIP protein binds to an ACGT-containing element (ACE) is mainly determined by the two nucleotide triplets 5' and 3' to the ACGT core of the sequence elements (Foster *et al.*, 1994).

Further analysis of one of the CPRF bZIP factors, CPRF1, revealed that it recognises the ACE from the parsley LRU1 both *in vitro* and *in vivo* (Armstrong *et al.*, 1992; Feldbrügge *et al.*, 1994). Feldbrügge *et al.* (1996) reported the cloning and sequencing of *CPRF1* genomic DNA. Analysis of this sequence data revealed the existence of 12 exons and 11 introns within a stretch of around 9 kb. Northern analysis revealed that *CPRF1* mRNA was present in all organs of light-grown plants in which *CHS* mRNA expression is detectable. A second RNA species which also hybridised to CPRF1 probes was identified as an alternatively spliced, additional *CPRF1* transcript

containing intron 8. This polyadenylated RNA species showed accumulation characteristics very similar to those of the *CPRF1* mRNA. Further, it was shown that *CPRF1* mRNA accumulation is light dependent and precedes the light-induced increase in *CHS* gene activity in both cultured parsley cells and intact parsley plants. The fact that *CPRF1* expression is light responsive is uncommon as most other characterised plant bZIP transcription factors appear to be under developmental control (Feldbrügge *et al.*, 1996; Foster *et al.*, 1994). Furthermore, Feldbrügge *et al.* (1996) also demonstrated that light-dependent *CHS* mRNA accumulation is blocked by cycloheximide. This indicates that translation of a protein factor, possibly, CPRF1, may be a prerequisite for *CHS* promoter activation.

Interestingly, the gene structure of *CPRF1* is such that exon 10 encodes the basic domain for DNA binding and exon 11 encodes the leucine zipper domain which is thought to direct dimerisation. The authors suggest that this organisation may imply that the basic region and the leucine zipper domain can be exchanged independently of other parts of the protein by exon shuffling. This exon structure is also seen in two other well characterised bZIP protein genes, *TGA1a* in tobacco and *TGA3* in Arabidopsis (Feldbrügge *et al.*, 1996; Fromm *et al.*, 1991; Miao *et al.*, 1994). More data on the gene structure of plant bZIP and other DNA-binding proteins will help to clarify this interesting aspect of evolution which may have contributed to the current range of transcription factors.

In 1997, Dröge-Laser *et al.* reported the identification of a novel bZIP factor, designated G/HBF1, from soybean. The cDNA encoding this factor was isolated from an expression library by screening with a 38 bp oligonucleotide containing a G-box and an H-box sequence found in the *PAL* and *CHS* promoters in soybean. The G-box is an ACGT-containing element and the H-box shows some similarity to the MYB recognition elements. This cDNA encoded an ORF of 1134 bp and the deduced amino acid sequence indicated a protein of around 41 kD in size which showed the

characteristic features of a bZIP protein. Interestingly, the carboxy-terminal half of the basic region of G/HBF1 shows similarity to many other bZIP basic regions but the amino-terminal half of the basic region shows similarity only to the parsley bZIP CPRF2 (Weisshaar *et al.*, 1991b). Recombinant G/HBF1 bound both G-box and H-box oligonucleotide sequences in gel shift assays. However, oligonucleotides containing both the G-box and H-box showed the same mobility as those containing only one box. This suggests that G/HBF1 binds at only one location when both *cis*-elements are in close proximity. Northern analysis and immunological studies revealed no notable expression pattern for G/HBF1 nor any nuclear/cytoplasmic translocation phenomena in cells treated with glutathione. Post-translational modification is therefore likely to play a role in the modulation of G/HBF1 activity. Analysis revealed that G/HBF1 becomes phosphorylated, mainly on serine residues, *in vivo* when soybean cell culture is treated with glutathione or pathogen. Further, *in vitro* phosphorylation of G/HBF1 was observed when treated with cytosolic extracts from cells elicited with glutathione or *P. syringae*. This resulted in markedly enhanced *cis*-element binding compared with G/HBF1 treated with extracts from unstimulated cells (Dröge-Laser *et al.*, 1997). This information suggests that the activity of this bZIP is modulated mainly at the level of phosphorylation.

An interesting and unexpected observation has been made concerning the intracellular distribution of bZIPs in plants. Approximately 90% of the *Arabidopsis* and soybean G-box binding activity, as well as the protein cross-reacting with antiserum made against the bZIP factor GBF1, is found to reside in the cytoplasm (Menkens *et al.*, 1995). The G-box is a member of the family of ACGT-containing elements and as such are bound by bZIP transcription factors. Further, it has been reported that transportation of the bZIP factor, GBF2, into the nucleus is selectively stimulated by blue light in *Arabidopsis* (Menkens *et al.*, 1995). This light-stimulated transportation is similar to that observed for the COP1 protein and it will be interesting to find out if the mechanisms of transportation are related.

It is well documented that phosphorylation plays a major role in modulating the activity of transcription factors (Hunter and Karin, 1992). Studies in *Arabidopsis* have shown that the DNA-binding activity of a recombinant bZIP, GBF1, is strongly enhanced by phosphorylation with casein kinase II prepared from broccoli (Klimczak *et al.*, 1992). Thus phosphorylation appears to play an important role in the light-regulated modulation of transcription factor activity in plants.

1.7.2 Plant MYB Transcription Factors

Families of transcription factors are often categorised by their DNA-binding domains. This is true for the family of transcription factors known as the MYB-like proteins. The best studied example of a protein containing this type of DNA-binding domain is the vertebrate activator of transcription, cMYB. This is a regulatory protein involved in proliferation and differentiation of haematopoietic cells. Its DNA-binding domain consists of three imperfect repeats, known as MYB repeats, which form helices, each approximately 50 amino acids in length which contain three regularly spaced tryptophan residues. In the case of cMYB, it was demonstrated that the second and third helices were both necessary and sufficient for DNA binding (Gabrielsen *et al.*, 1991; Howe *et al.*, 1990; Saikumar *et al.*, 1990). Members of the MYB-like family of transcription factors are those which have at least one MYB repeat in their structure. The majority of MYB-like proteins in plants possess a DNA-binding domain with a two MYB repeat structure similar to the second and third helices of cMYB (reviewed by Solano *et al.*, 1995).

A MYB-like protein, PcMYB1 has been cloned and sequenced from parsley (Feldbrügge *et al.*, 1997). Using gel shift assays and cotransfection experiments, PcMYB1 has been shown to specifically bind the MYB recognition element from the parsley *CHS* promoter *in vitro* and *in vivo*. The deduced amino acid sequence revealed

that PcMYB1 contains only one MYB-like repeat. This portion of the protein constitutes the DNA-binding domain. Mutational analysis of PcMYB1, in combination with sequence comparison, suggests the presence of a helix-turn-helix structure containing a recognition helix which is sufficient for sequence-specific binding. Interestingly, the structure of this distinct MYB-like DNA-binding domain appears to be conserved in proteins from all three eukaryotic phyla (Feldbrügge *et al.*, 1997).

Northern analysis revealed that the single copy *PcMYB1* gene seems to be neither activated by light nor organ specifically expressed (Feldbrügge *et al.*, 1997). The authors suggest that, for light-mediated gene activation, PcMYB1 might be post-transcriptionally regulated, act in concert with other light-induced transcription factors, for instance CPRF1, or perhaps both of these regulatory mechanisms occur. One might speculate that nuclear translocation or phosphorylation might also be good candidates for regulation of PcMYB1 activity.

A European project is currently underway to clone and characterise the MYB-like transcription factors in *Arabidopsis*. As part of this project, Weisshaar and co-workers have identified a protein, designated AtMYB44 which is capable of binding to the MYB recognition element from the *CHS*, chalcone flavone isomerase (*CFI*) and flavonol synthase (*FLS*) promoters from *Arabidopsis in vitro*. These genes are all involved in flavonoid biosynthesis and are thought to be co-regulated (personal communication with Dr. B. Weisshaar). Further analysis will reveal whether this factor is also capable of binding the *CHS* MRE *in vivo* and thus indicate whether it is likely to be involved in the regulation of *CHS* gene activity in *Arabidopsis*.

1.7.3 Interaction of Different Classes of Transcription Factors

The activation of the promoters of the genes involved in anthocyanin biosynthesis in maize is well studied (Mol *et al.*, 1996). Regulation of this anthocyanin biosynthesis

pathway requires two classes of transcription factors. One class of regulators contains a MYB domain (for example, C1 and P1) and the other class contains a basic-helix-loop-helix domain (for instance, B and R) (Goff *et al.*, 1990). These genes were identified by genetic analysis which also revealed that a protein from each class must be expressed to activate the genes of the anthocyanin biosynthesis pathway; neither alone is sufficient for induction. The C1 and B proteins directly interact with one another in the two-hybrid assay suggesting that these proteins physically act together to activate the genes of this pathway (Goff *et al.*, 1992).

The precise role of the B protein in activating the anthocyanin biosynthesis genes is uncertain. Studies have not yet revealed either specific DNA-binding or an activation domain (Lesnick and Chandler, 1998; Goff *et al.*, 1992). In contrast, the C1 protein binds via its MYB domain to the promoter of the *A1* anthocyanin biosynthesis gene, which is normally referred to as the dihydroflavonol reductase (*DFR*) gene, and contains an acidic activation domain (Sainz *et al.*, 1997; Goff *et al.*, 1991). Taken together with the observation that C1 and B physically interact, it appears that these proteins directly activate transcription of the biosynthetic genes of the pathway. The promoters of several maize genes, for instance *A2* and *BRONZE2*, contain a "C1-motif" and a "R-motif" which are thought to be important for co-activation by MYB-like (C1-related) and bHLH (R-like) transcription factors (Lesnick and Chandler, 1998; Bodeau and Walbot, 1996).

Recent analysis has indicated that a similar mechanism of regulation exists in French bean. Faktor *et al.* (1997a/1997b) reported that a 39 bp region of the *CHS15* promoter, containing G-box and H-box elements, directed both tissue-specific expression and stress-responsive activation in transgenic tobacco plants. The H-box shares similarity with the C1-motif and may act as a MYB recognition element. As previously mentioned, the G-box belongs to a large family of ACGT-containing elements which are generally recognised by bZIP factors. Several different studies have revealed three

types of sequence elements which are commonly found together. These are R-motifs, bHLH factor binding sites which are related to ACEs, and MREs. It has been suggested that the combinatorial interaction of a MRE with an ACE, or a MRE with a R-motif, may well provide specificity in the developmentally- and stimulus-dependent gene activation process.

The amount of information available on these regulators is increasing rapidly. Biochemical studies in parsley, bean/tobacco and *Arabidopsis* and genetic data from maize, petunia and snapdragon have contributed greatly to this (reviewed by Mol *et al.*, 1996). The MYB-like protein, C1, has been identified as a regulator of anthocyanin biosynthesis both biochemically and genetically (Sainz *et al.*, 1997; Goff *et al.*, 1990). Genetic studies have implicated MYBs and bHLH proteins in regulating pigment formation whereas a biochemical approach has suggested a role for MYBs and bZIP proteins in transcriptional activation. There is no biochemical data supporting the involvement of bHLH factors nor is there genetic evidence implicating bZIPs in transcriptional regulation. It seems unlikely, given the highly conserved nature of transcriptional machinery, that this contradiction is due to differences between species. It might be that there is a more fundamental difference between the genetic analyses in maize and the biochemical studies performed in other species. One such difference is that the genetic analysis has focused on tissue-specific and developmental expression of genes involved in pigment formation, whereas in the biochemical systems stimulus-dependent expression, usually in response to light or elicitor, has been studied. One hypothesis to explain these observations is that the bZIP factors act specifically in stimulus-dependent gene activation, whilst bHLH factors activate gene expression in a cell-identity dependent manner. In both situations, these factors would interact with MYB-like factors to regulate gene expression (Weisshaar and Jenkins, 1998).

1.8 CONCLUSIONS

In recent years considerable progress has been made in understanding the mechanisms involved in light-regulated plant development. A combination of biochemical and genetic approaches has unveiled important insights into plant photosensory perception and signal transduction. As with the phytochromes, several UV/blue photoreceptors appear to exist in plants. Although primary phytochrome signal transduction events have been identified, much less is known about the upstream signalling events following UV/blue photoreception. Together with the traditional pharmacological approach, continuing biochemical analysis of the *COP/DET/FUS* mutants will reveal some of the essential light signalling components and hopefully help us to understand the nature of the crosstalk among different regulatory pathways. As the number of reported plant transcription factors grows, it becomes increasingly more important to link them to specific stimulus-dependent or developmental responses. It is therefore essential to develop systems in which the mechanisms coupling UV/blue light perception to defined downstream responses, in particular gene expression, can be dissected. The continued integration of biochemical and genetic approaches is central to understanding the nature of UV/blue light regulation of gene expression.

1.9 PROJECT AIMS

The overall aim of this project was to develop a system in *Arabidopsis* to analyse UV/blue light regulation of transcription. The single *CHS* gene of *Arabidopsis* provides an excellent system to investigate the cellular and molecular mechanisms through which specific UV and blue light receptors control transcription. In contrast to other species phytochrome appears to have little involvement in the regulation of *CHS* in *Arabidopsis* (Kaiser *et al.*, 1995; Batschauer *et al.*, 1996) whereas expression is induced by UV-B

and UV-A/blue light (Li *et al.*, 1993; Jackson *et al.*, 1995; Fuglevand *et al.*, 1996). The development of a light-responsive homologous protoplast transient expression system was undertaken in order to address the following questions:

- (i) What are the *cis*-acting elements of the *A. thaliana* *CHS* promoter concerned with light regulation ?
- (ii) Which elements are specifically concerned with regulation by UV-A/blue and which are concerned with UV-B light?
- (iii) Which of the known photoreceptors play a role in the control of *CHS* gene expression and how is the photoreceptor action integrated?
- (iv) How is the light regulation of *AtCHS* transcription integrated with metabolic regulation? It is known that sugars also stimulate *CHS* transcription.

An *Arabidopsis* cell culture system (Christie and Jenkins, 1996), in which the UV-B and UV-A/blue light regulation of *CHS* gene expression resembles that in mature leaf tissue (Jackson *et al.*, 1995), was used to develop the protoplast transient expression system. Experiments were undertaken to optimise this system. Subsequently, several different *CHS* promoter: GUS fusions were made to dissect the regions of the promoter concerned with UV-B and UV-A/blue light regulation. The results of this research have been published (Hartmann, U., Valentine, W.J., Christie, J.M., Hays, J., Jenkins, G.I. and Weisshaar, B. Identification of UV/blue light-response elements in the *Arabidopsis thaliana* chalcone synthase promoter using a homologous protoplast transient expression system. *Plant Molecular Biology* **36**, 741 - 754, 1998). To address question (iii), co-transfection experiments with cryptochrome fusions were employed. These "gain of function" assays were complemented with experiments with *Arabidopsis* photoreceptor mutants. Finally, the transient expression system was used to address the role of sugars in regulating *AtCHS* expression.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The chemicals used in this study were obtained from BDH (AnalaR[®] grade, Poole, UK) unless otherwise indicated. The sugars used in the experiments described in chapter 6 were obtained from Sigma (Poole, UK).

2.1.2 Radiochemicals

The radiochemicals used in this study were supplied by Amersham International (Amersham, UK).

2.1.3 Plasmids and Bacterial Strains

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

2.1.4 Liquid and Solid Bacterial Growth Media

TYN medium (1 % (w/v) bacto-tryptone, 1 % (w/v) bacto-yeast extract (both supplied by Merck, Darmstadt, Germany), 0.5 % (w/v) NaCl, supplemented with the appropriate antibiotic, was used for the culture of *E.coli*. For the plating out of *E.coli* TYN-agar was prepared by adding 1.5 g/l agar (Sigma) to TYN media prior to sterilisation. The appropriate antibiotic was added after sterilisation when the solution had cooled to 50 °C.

Figure 2.1 Plasmid DNA used in this study

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

Plasmid	Description	Reference
pCHS	<i>Arabidopsis CHS</i> 1059 bp <i>Hind</i> III genomic DNA fragment in pUC19	Trezzini <i>et al.</i> (1993)
pH1	<i>Phaseolus vulgaris H1</i> 1.4 kb <i>Pst</i> I cDNA fragment in pAT153	Lawton and Lamb (1987)
p35S-GUS	<i>CaMV</i> 35S promoter-GUS fusion in pBT2	Weisshaar <i>et al.</i> (1991b)
pBT2	Promoterless-GUS coding sequence in pUC19	Weisshaar <i>et al.</i> (1991b)
pAtCHS -1972	Full-length <i>AtCHS</i> promoter-GUS fusion in pBT2	Hartmann <i>et al.</i> (1998)
pAtCHS -1972 MREm	Full-length <i>AtCHS</i> promoter containing a block mutation in MRE-GUS fusion in pBT2	Hartmann <i>et al.</i> (1998)
pAtCHS -1972 ACEm	Full-length <i>AtCHS</i> promoter containing a block mutation in ACE-GUS fusion in pBT2	Hartmann <i>et al.</i> (1998)
pAtCHS -1972 dm	Full-length <i>AtCHS</i> promoter containing block mutations in MRE and ACE-GUS fusion in pBT2	Produced during this study
pAtCHS -1416	-1416 bp deletion <i>AtCHS</i> promoter-GUS fusion in pBT2	Hartmann <i>et al.</i> (1998)
pAtCHS -1416 dm	-1416 bp deletion <i>AtCHS</i> promoter containing block mutations in MRE and ACE-GUS fusion in pBT2	Produced during this study
pAtCHS -668	-668 bp deletion <i>AtCHS</i> promoter-GUS fusion in pBT2	Hartmann <i>et al.</i> (1998)
pAtCHS -668 dm	-668 bp deletion <i>AtCHS</i> promoter containing block mutations in MRE and ACE-GUS fusion in pBT2	Produced during this study
pAtCHS -668 gm	-668 bp deletion <i>AtCHS</i> promoter containing a block mutation in G-box-like element-GUS fusion in pBT2	Hartmann <i>et al.</i> (1998)

pAtCHS -668 tm	-668 bp deletion <i>AtCHS</i> promoter containing block mutations in the MRE, ACE and G-box-like element-GUS fusion in pBT2	Hartmann <i>et al.</i> (1998)
pAtCHS -336	-336 bp deletion <i>AtCHS</i> promoter-GUS fusion in pBT2	Hartmann <i>et al.</i> (1998)
pAtCHS -336 dm	-336 bp deletion <i>AtCHS</i> promoter containing block mutations in MRE and ACE-GUS fusion in pBT2	Produced during this study
pAtCHS -164	-164 bp deletion <i>AtCHS</i> promoter-GUS fusion in pBT2	Hartmann <i>et al.</i> (1998)
pAtCHS -164 MREm	-164 bp deletion <i>AtCHS</i> promoter containing a block mutation in MRE-GUS fusion in pBT2	Hartmann <i>et al.</i> (1998)
pAtCHS -164 ACEm	-164 bp deletion <i>AtCHS</i> promoter containing a block mutation in ACE-GUS fusion in pBT2	Produced during this study
pAtCHS -164 dm	-164 bp deletion <i>AtCHS</i> promoter containing block mutations in MRE and ACE-GUS fusion in pBT2	Produced during this study
pAtCHS LRU-4	<i>Arabidopsis</i> LRU tetramer fused to 35S core promoter-GUS fusion in pBT2	Hartmann <i>et al.</i> (1998)
pAtCHS -61	-164 bp deletion <i>AtCHS</i> promoter containing a block mutation in ACE-GUS fusion in pBT2	Hartmann <i>et al.</i> (1998)

2.1.5 Antibiotics

The antibiotics used were supplied by Sigma. Ampicillin was dissolved in distilled water (25 mg/ml), filter-sterilised and used at a final concentration of 50 µg/ml. Kanamycin was also dissolved in distilled water (25 mg/ml), filter-sterilised and used at a final concentration of 50 µg/ml.

2.1.6 DNA Modifying Enzymes

All DNA modifying and restriction enzymes were purchased from Promega (Southampton, UK) together with their reaction buffers which were provided at 10 x concentration unless otherwise indicated.

2.2 General Laboratory Procedures

2.2.1 pH Measurement

The pH of solutions was measured using a Jenway pH meter 3320 and a Gelplas combination electrode (BDH).

2.2.2 Autoclaving

Equipment and solutions were sterilised at 15 psi for 20 min in a Laboratory Thermal Equipment Autoclave 225E. Cell culture media was autoclaved under the same conditions for 30 min.

2.2.3 Filter Sterilisation

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

2.2.4 Solutions and Equipment for RNA Work

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

2.3 Plant Material

2.3.1 Growth and Harvesting of Plants

Seeds of *Arabidopsis thaliana* cv Landsberg erecta wild-type and *hy4-2.23N* mutant seeds and *fha1.1* seeds were obtained from the *Arabidopsis* Stock Centre (Nottingham, UK). *Arabidopsis* seeds were stored at room temperature and germinated in pots containing water soaked ICI potting compost. Pots were covered with clingfilm and placed in the dark for 3 to 4 days at 4 °C in order to break seed dormancy. Plants were then grown in low intensity white light ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) at 20 °C unless otherwise indicated. For the isolation of RNA, tissue was harvested directly into liquid nitrogen and stored at -80 °C until use.

2.3.2 Growth of *Arabidopsis* Cell Cultures

The light-grown *At-glw Arabidopsis* cell and callus cultures were supplied by Dr M. May (Dept. of Plant Sciences, University of Oxford) and have been described previously (May and Leaver, 1993). The light-grown *hy4* cell and callus cultures were supplied by Dr J.M. Christie and have also been described previously (Christie, 1996). Both cultures were grown in the same way. Cells were grown photomixotrophically in 200 ml culture media containing Murashige-Skoog salts, 0.5 mg/l α -naphtheleneacetic acid, 0.05 mg/l kinetin (all obtained from Sigma), 3 % (w/v) sucrose, pH 5.8 in 500 ml conical flasks. Suspension cultures were grown at 20 °C in continuous low intensity white light ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) with constant shaking (110 rpm). The cells were subcultured every week by a 1/10 dilution. Callus cultures were grown in culture media

supplemented with 0.8 % (w/v) agar (Sigma) and subcultured to fresh growth media every 2 weeks.

The dark-grown *At-7 Arabidopsis* cell culture was supplied by Dr B. Weisshaar (Max Planck Institute, Cologne) and has been described previously (Trezza et al., 1993). *At-7* cells were grown heterotrophically in 40 ml culture media containing Murashige-Skoog salts (Sigma), 1 mg/ml 2,4-D, 100 mg/l inositol, 1 mg/l thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, 3 % (w/v) sucrose (pH 5.7) in 250 ml conical flasks. The suspension culture was grown at 26 °C in the dark with constant shaking (110 rpm). *At-7* cells were subcultured every week by aseptically transferring 4 g of the 1 week old culture to a separate flask containing 40 ml fresh culture media. On the fifth day after subculturing, cells were used for protoplast isolation.

2.3.3 Monitoring Cell Culture Contamination

Bacterial and fungal contamination of the *At-glw* and *hy4* cell cultures was monitored periodically (every 3 months). Cell aliquots (2 ml) were aseptically transferred to 9 cm Petri dishes containing 1.5% (w/v) nutrient agar (Sigma) or czapek-dox agar (1x czapek-dox broth (Sigma), 1.5% (w/v) agar) and the plates sealed with Micropore® tape (3M Health Care, Loughborough, UK). The plates were placed at 37°C overnight and then examined for contamination. In the event of bacterial or fungal growth cell cultures were discarded. If necessary, new cultures were established from friable callus.

2.4 Illumination of Plant Material

2.4.1 Light Sources

Illuminations were carried out in controlled environment rooms at 20 °C. The spectra of all of the light qualities were measured with a spectroradiometer (Macam SR9910) and

are shown in Figure 2.2. White light was provided by warm white fluorescent tubes (Osram, Munich, Germany), UV-A blue light was provided by Sylvania 40 W T12 blue fluorescent tubes (GTE Sylvania, Shipley, UK) and red light was obtained by covering the white fluorescent tubes with orange Cinemoid. UV-A light was provided by Sylvania F35W/B1-2B blacklight-blue fluorescent tubes emitting wavelengths between 320 and 390 nm. UV-B light was obtained by covering Phillips TL 40W/12 ultra-violet fluorescent tubes with cellulose acetate (to omit UV-C), which was changed every 24 h. The required fluence rate was achieved by varying the number of tubes in the growth area and adjusting the distance of the plant material from the light source. In the appropriate experiments, wavelengths below 320 nm were removed by covering tubes with a clear polyester 130 filter (Lee Filters, Andover, UK). Wavelengths below 390 nm were removed using a clear polyester 226 filter (Lee filters).

2.4.2 Fluence Rate Measurement

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

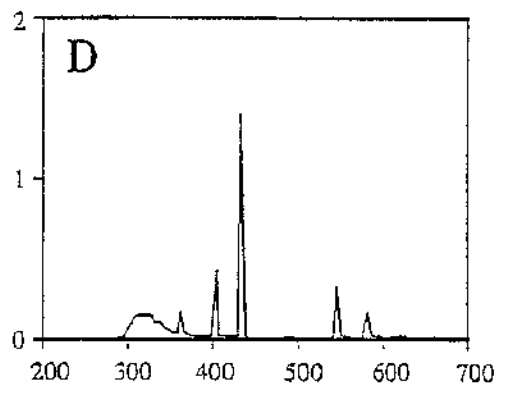
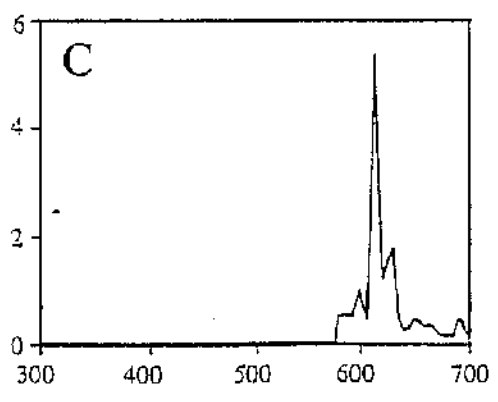
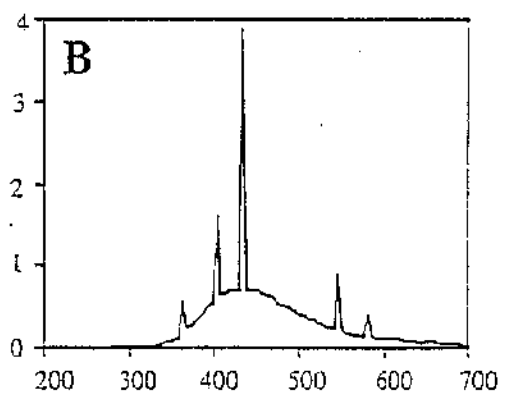
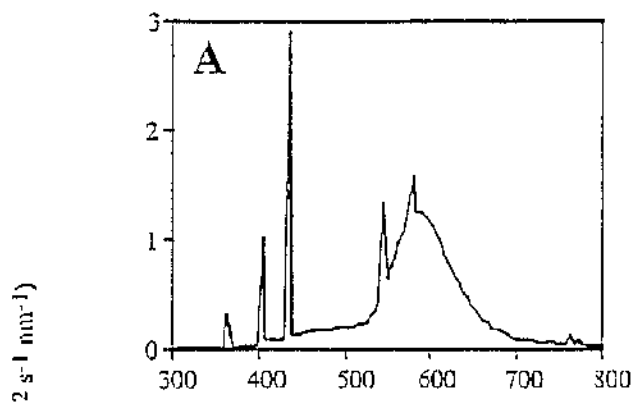
2.5 Morphological Measurements

Measurements of all hypocotyl lengths were made with a ruler on 8 day old seedlings. In order to estimate cotyledon area, cotyledons were removed from 7 day old seedlings and stuck onto clear sticky tape. The tape was placed on an overhead projector and the image magnified and traced onto paper of a standard thickness. The enlarged images were cut out and weighed relative to standard areas thus giving an estimate of cotyledon size.

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

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

- (A) White ($120 \mu\text{mol.m}^{-2}.\text{s}^{-1}$)
- (B) UV-A/blue ($80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$)
- (C) Red ($80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$)
- (D) UV-B ($3 \mu\text{mol.m}^{-2}.\text{s}^{-1}$)



Wavelength (nm)

2.6 Isolation of Total RNA from Plant Material

Approximately 0.5 g of frozen leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle and transferred to a 1.5 ml Eppendorf[®] tube. Total RNA was then extracted using the PUREscript[®] RNA Isolation Kit (Flowgen, Lichfield, UK) according to the manufacturers instructions. 300 µl of cell lysis solution (containing citric acid, EDTA and SDS) was added to the tube and then vortexed. 100 µl of protein/DNA precipitation solution (containing citric acid and NaCl) was then added and mixed gently. After mixing, samples were incubated on ice for 5 min and microcentrifuged for 5 min. The supernatant was transferred to clean 1.5 ml Eppendorf[®] tube containing 300 µl of *iso*-propanol and mixed by inversion before microcentrifugation for a further 5 min. The supernatant was then discarded and the pellet allowed to dry before washing in 70 % (v/v) ethanol. Samples were microcentrifuged for 1 min and the resulting pellet dried and resuspended in an appropriate volume of DEPC-treated dH₂O.

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

2.7 Amplification and Preparation of Plasmid DNA

2.7.1 Preparation of Competent Cells

Competent cells were prepared by the method of Sambrook *et al.* (1989). *E.coli* was grown overnight in 10 ml TYN media at 37 °C with constant shaking. 100 ml of fresh TYN media was inoculated with 5 ml of the overnight culture and shaken at 37 °C until an OD at 550 nm of 0.35 was reached. 70 ml of the suspension was transferred to two sterile 35 ml centrifuge tubes and chilled on ice for 15 min. Cells were pelleted at 2,000

g for 5 min at 4 °C and the supernatant decanted. The bacterial pellet was resuspended in a total volume of 10.5 ml ice-cold TFB 1 (100 mM RbCl, 50 mM MnCl₂, 30 mM KOAc, 10 mM CaCl₂, 15 % (v/v) glycerol) and incubated on ice for 90 min. After incubation, the cell suspension was centrifuged as before and resuspended in 2.8 ml ice-cold TFB 2 (10 mM MOPS (Sigma), 10 mM RbCl, 75 mM CaCl₂, 15 % (v/v) glycerol (pH 7.0)). Cells were then separated into 0.2 ml aliquots, in 1.5 ml Eppendorf® tubes, frozen in liquid nitrogen and stored at -80 °C until use.

2.7.2 Transformation of Competent Cells

Frozen competent cells (2.7.1) were thawed at room temperature and placed on ice. Approximately 200 ng of plasmid DNA, in a volume of 5 - 10 µl, was added to an aliquot of competent cells and the tube gently shaken and returned to ice for 20 min. After this period, cells were heat-shocked at 37 °C for 1 min and returned to ice for a further 2 min. TYN media (0.8 ml) was added to the cells and the tube shaken at 37 °C for 50 min to allow expression of the antibiotic resistance encoded on the plasmid. Cells were then pelleted by centrifugation at 10,000 g for 30 s, the supernatant discarded, and the pellet resuspended in 0.1 ml of TYN medium. 100 µl serial 1/10 dilutions of the cell suspension were plated out on agar plates with the appropriate antibiotic. The plates were allowed to dry and placed at 37 °C overnight. As a control, transformed cells were plated on agar in the absence of antibiotic selection. A second control was also performed where competent cells were treated as above, in the absence of plasmid DNA and plated on antibiotic-containing plates.

2.7.3 Small Scale Preparation of Plasmid DNA

For this method the Wizard™ Minipreps DNA Purification System (Promega) was used in accordance with the manufacturer's instructions. A single bacterial colony was used to inoculate 3.0 ml of TYN medium, supplemented with the appropriate antibiotic, in a 7 ml bijou tube. The culture was grown overnight with constant shaking at 37 °C. 1 ml of the overnight culture was transferred to a 1.5 ml Eppendorf® tube and

centrifuged at 10,000 g for 5 min. The supernatant was discarded and the bacterial pellet resuspended by vortexing in 200 μ l of cell resuspension solution (50 mM Tris-HCl pH 7.5, 10mM EDTA, 100 mg.ml⁻¹ RNase A). Once fully resuspended, 200 μ l of cell lysis solution (0.2 M NaOH, 1% SDS) was added and mixed by gentle inversion. Next, 200 μ l of neutralisation solution (1.32 M KOAc pH 4.8) was added and mixed as before. The tube was centrifuged at 10,000 g for 5 min after which the supernatant was removed to a 2.0 ml Eppendorf[®] tube. 1 ml of the Wizard[™] Minipreps DNA purification resin was added and the solution gently mixed. The resin/DNA mixture was then transferred to a 3 ml disposable syringe, attached to a minicolumn and placed on a vacuum manifold (Promega). A vacuum was then applied to pull the slurry into the minicolumn. The vacuum was broken, 2 ml of wash solution (100 mM NaCl, 10 mM Tris-HCl pH7.5, 2.5 mM EDTA, 55 % (v/v) ethanol) added and the vacuum reapplied. Once the wash solution had passed through, the column was left to dry for 2 min. The vacuum was broken and the minicolumn removed from the syringe and transferred to a 1.5 ml Eppendorf[®] tube. This was microcentrifuged for 20 s to further dry the resin. The minicolumn transferred to a clean 1.5 ml Eppendorf[®] tube, 50 μ l of TE (pH 7.6) added and allowed to stand for 1 min at room temperature. Plasmid DNA was eluted by microcentrifugation for 20 s and the minicolumn discarded.

2.7.4 Large Scale Preparation of Plasmid DNA

Plasmid DNA for transient gene expression studies was amplified using bacterial cultures (2.7.4.1) and purified using the Qiagen mega-plasmid purification system (2.7.4.2) (Qiagen, UK). After purification, plasmid DNA was analysed by digestion (2.11) with the appropriate restriction enzymes prior to use in transient gene expression assays.

2.7.4.1 Growth of Bacterial Cultures

A single bacterial colony containing the plasmid of choice was used to inoculate 10 ml of TYN medium, containing the appropriate antibiotic, and grown overnight at 37 °C

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

2.7.4.2 Preparation of Plasmid DNA using the Qiagen Mega-Plasmid Purification System

The Qiagen mega-plasmid purification system was used in accordance with the manufacturer's instructions. Cells containing the plasmid of choice were prepared as described in 2.7.4.1. The cell pellet was resuspended in 50 ml P1 buffer (100 mg.ml⁻¹ RNase A, 50 mM Tris-HCl, 10 mM EDTA (pH 8.0)) and transferred to a clean centrifuge tube. 50 ml of P2 buffer (200 mM NaOH, 1 % (w/v) SDS) was added to the cell suspension and the solution mixed gently by inverting the bottle several times before incubating at room temperature for 5 min. Next, 50 ml of P3 buffer (3 M KOAc (pH 5.5)) was added and the solution incubated on ice for 30 min after mixing by inversion. The suspension was then centrifuged at 4 °C for 5 min at 20,000 g. Meanwhile, a Qiagen column 2,500 was equilibrated by applying 35 ml QBT buffer (750 mM NaCl, 50 mM MOPS, 15 % (v/v) ethanol, 0.15 % (v/v) Triton X-100 (pH 7.0)) and the column allowed to empty by gravity flow. The supernatant obtained after centrifugation was then applied to the column and allowed to enter the resin by gravity flow. The column was washed with 4 x 50 ml QC buffer (1 M NaCl, 50 mM Tris-HCl, 15 % (v/v) ethanol (pH 8.5)). Plasmid DNA was eluted into a sterile 200 ml centrifuge bottle by the addition of 35 ml QF buffer (1.25 NaCl, 50 mM Tris-HCl, 15 % (v/v) ethanol (pH 8.5)). DNA was precipitated by adding 0.7 volumes of *iso*-propanol, the solution mixed, transferred to 2 x 35 ml centrifuge tubes and spun at 20,000 g for 30 min at 4 °C. The supernatant was discarded and DNA pellets washed in 5 ml of ice cold 70 % (v/v) ethanol and centrifuged once more. The supernatant was decanted and the pellets air-dried before redissolving in a suitable volume of TE buffer (pH 7.6). The

concentration of the DNA solution was determined as described in 2.8 and diluted to the required concentration before storing at 4 °C.

2.8 Quantification of DNA and RNA

An aliquot of the nucleic acid solution to be quantified (usually 5 - 10 µl) was made up to 1 ml with DEPC-treated dH₂O. The absorbance of the solution was measured between 220 nm and 320 nm against a DEPC-treated dH₂O blank. An absorbance at 260 nm of 1 was taken to indicate the following concentrations:

<u>Form of nucleic acid</u>	<u>Concentration (mg.ml⁻¹)</u>
Double stranded DNA	50
Single stranded DNA and RNA	40

2.9 Gel Electrophoresis

All agarose (ultra PURE™) was supplied by Life Technologies. Acrylamide was supplied by Merck.

2.9.1 Agarose Gel Electrophoresis of DNA

The appropriate concentration of agarose (0.5 - 2.0 % (w/v)) was added to the volume of 1 x TAE (0.09 M Tris-acetate, 2 mM EDTA) required for the gel being cast. The agarose suspension was heated in a microwave oven until the agarose had completely dissolved. The solution was allowed to cool to around 60 °C at which point 10 mg.ml⁻¹ EtBr was added to a final concentration of 1 mg.ml⁻¹ and the gel solution poured into the electrophoresis apparatus. After allowing the gel to set for 30 min, enough 1 x TAE running buffer was added to just submerge the gel. The samples to be loaded were mixed with 1/10 volume of loading buffer (20 % (v/v) glycerol, 25 mM NaOH, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF) and loaded with an

automatic pipette. Electrophoresis was carried out at 15 - 80 mA until the bromophenol blue had migrated two-thirds of the way down the gel. The gel was then visualised under UV light (Spectrolite® transilluminator, Model TC-312A).

2.9.2 Polyacrylamide Gel Electrophoresis of DNA

Polyacrylamide gel electrophoresis of DNA was performed according to the method of Sambrook *et al.* (1989). The gel was made by dissolving 33.6 g urea in 30 ml dH₂O and adding 16 ml 40 % acrylamide solution (63.3 % (w/v) acrylamide, 3.3 % (w/v) *N, N'* - methylenebisacrylamide), 8 ml 10 x TBE buffer (890 mM tris-borate, 2 mM EDTA), 480 µl 10 % (w/v) ammonium persulphate and 60 µl TEMED (*N, N, N', N'* - tetramethylethylenediamine) before pouring in between the plates of the gel apparatus. The gel apparatus was assembled and 1 x TBE buffer was added as a running buffer. Denatured DNA samples were loaded and the gel run at approximately 70 °C and 85 watts (150 mA) for around 3 h. The gel was soaked in 10 % acetic acid, rinsed with dH₂O and dried at 80 °C for 1 h. The gel was then visualised by autoradiography (2.18.3).

2.9.3 Denaturing Electrophoresis of RNA

This method was used for gels that were to be northern blotted for hybridisation analysis. The appropriate amount of agarose (1.3 - 1.5 g) was added to 80 ml of DEPC-treated dH₂O. The agarose suspension was then heated in a microwave oven until the agarose had completely dissolved. Once the agarose solution had cooled to 60 °C, 10 ml of formaldehyde (37 % (v/v), Sigma) and 10 ml 10 x MOPS buffer (0.2 M MOPS, (Sigma), 0.05 M NaOAc, 0.01 M EDTA (pH 7.0)) was added and the gel mixed by swirling prior to pouring into the electrophoresis apparatus. After a period of 30 min the gel had set and was transferred to 4 °C for another 30 min. After this, the gel was placed into the electrophoresis tank and submerged in 1 x MOPS. RNA (5 - 20 µg) was prepared in a solution of 70 % (v/v) formamide (Fluka Biochemicals, Gillingham, UK), 2.3 x MOPS, 2.3 % formaldehyde to a volume no greater than 50

μ l. This solution was heated to 65°C for 5 min and placed on ice. One-tenth volume of loading buffer (50 % (v/v) glycerol, 1 mM EDTA (pH 8.0), 0.25 % (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF) was then added and the samples loaded onto the gel with a pipette. Electrophoresis was carried out at 15 - 80 mA until the bromophenol blue had migrated two thirds of the distance down the gel. The gel was then visualised under UV light.

2.10 Isolation of DNA Fragments from Agarose Gels

2.10.1 DNA Extraction using Cellulose Nitrate Filters

The DNA fragment of interest was initially separated from residual DNA by agarose gel electrophoresis as described in 2.9.1 and identified by EtBr staining when viewed under UV light. The fragment was then excised with a clean scalpel blade, cut into small pieces and transferred onto a cellulose nitrate filter (Sartorius, Germany) soaked in dH₂O inside a 1.5 ml Eppendorf® tube with a small hole in the bottom. This was placed into a second 1.5 ml Eppendorf® tube and microcentrifuged for 5 min. The upper tube was discarded, an equal volume of phenol added to the sample and vortexed. The sample was microcentrifuged for 5 min and the upper phase was removed to a clean tube. An equal volume of chloroform was added and the solution briefly vortexed. The sample was again microcentrifuged for 5 min. The upper layer was removed to a clean tube and 1/10 volume of 3 M NaOAc (pH 5.5) added along with 2.5 volumes of ethanol and then microcentrifuged for 20 min. The supernatant was then discarded and the pellet washed with 70 % (v/v) ethanol before microcentrifuging for 5 min. The supernatant was removed, the pellet air-dried for 10 min then resuspended in an appropriate volume of TE buffer (pH 7.6).

2.10.2 Qiaex Gel Extraction of DNA

For this method the Qiaex Gel Extraction System (Qiagen) was used in accordance with the manufacturer's instructions. The DNA of interest was isolated from the agarose gel as described in 2.10.1. The gel slice was transferred to a 1.5 ml Eppendorf[®] tube, containing 300 µl buffer QX1 (3 M NaI, 4 M NaClO₄, 10 mM Tris-HCl (pH 7.0), 10 mM sodium thiosulphate). The sample was incubated at 50 °C until the agarose had completely dissolved. 10 ml Qiaex resin was added and the sample incubated at 50 °C for 10 min to allow the DNA to bind to the resin. After incubation, the sample was microcentrifuged for 30 s and the supernatant removed. The pellet was resuspended in 0.5 ml buffer QX2 (8 M NaClO₄, 10 mM Tris-HCl (pH 7.0)) and the sample centrifuged once more. This wash step was repeated a twice more with an equal volume of buffer QX3 (70 % (v/v) ethanol, 100 mM NaCl, 10 mM Tris-HCl (pH 7.5)). The final pellet was air-dried for 10 min and resuspended in 20 µl TE (pH 7.6) then incubated at room temperature for 5 min, vortexing periodically, to elute the DNA from the Qiaex resin. The sample was centrifuged as before and the supernatant transferred to clean tube.

2.11 Digestion of DNA with Restriction Endonucleases

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

2.12 DNA Ligations

Purified DNA restriction fragments were ligated based on the method of Sambrook *et al.* (1989). 0.1 µg of vector DNA was transferred to a sterile Eppendorf[®] tube and an

equimolar amount of insert DNA added. To the tube, 1 μ l of 10 x T4 DNA ligase buffer (200 mM Tris.Cl (pH 7.6), 50 mM MgCl₂, 50 mM dithiothreitol), 1 μ l 5 mM ATP and 1 unit T4 DNA ligase (Promega) was added. This mixture was diluted to 10 μ l with dH₂O. The samples were incubated either at room temperature for 2 h or overnight at 16 °C. Control reactions containing only vector DNA or only insert DNA were also set up. Ligations were directly transformed into competent *E. coli* (2.7.2) or analysed by agarose gel electrophoresis (2.9.1).

2.13 Klenow 5' Overhang Fill-In of DNA Restriction Fragments

Purified DNA restriction fragments with 5' overhangs were filled-in based on the method of Sambrook *et al.* (1989). 10 μ g of DNA was transferred to a sterile 1.5 ml Eppendorf® tube and 5 μ l 10 x Klenow buffer (10 mM Tris.Cl (pH 8.0), 0.1 M dithiothreitol) added. 10 μ l of nucleotide buffer (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP) and 3 units of Klenow enzyme (fragment of DNA polymerase) (Promega) was added. The sample was then diluted to 50 μ l with dH₂O and incubated at room temperature for 1 h and then at 65 °C for 5 min. Samples were purified as described in 2.10.1 or 2.10.2.

2.14 DNA Sequencing

All new DNA sequences were verified by sequencing both strands. Sequencing was performed using the Sequenase 2.0 kit (USB, USA) in accordance with the manufacturer's instructions. 2 μ g of the DNA to be sequenced was denatured with 2 μ l of 2 M NaOH and incubated at room temperature for 10 min. 3 μ l of 3 M NaOAc (pH 4.5) and 60 μ l ethanol were added and the sample microcentrifuged at 4 °C for 25 min. The supernatant was discarded and the pellet air-dried for 5 min before being resuspended in 10 μ l dH₂O. Primers were annealed by adding 80 ng of primer and 2 μ l annealing buffer (USB) and incubating at 37 °C for 20 min. To the sample 2 μ l

dH₂O, 6 µl label A mix (USB), 1 µl Sequenase (USB), 3 µl enzyme dilution buffer (USB) and 2 µl [³⁵S] dATP (Amersham) was added before incubation at room temperature for 10 min. The sample was then split into 4 equal aliquots and 2.5 µl of each ddNTP (USB) was added before incubation at 37 °C for 5 min. Reactions were stopped by adding 5 µl of loading buffer (20 % (v/v) glycerol, 25 mM NaOH, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF). Sequencing reactions were visualised by polyacrylamide gel electrophoresis (2.9.2) and autoradiography (2.18.3). The analysis of DNA sequences was performed using the GCG program package (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, USA).

2.15 Polymerase Chain Reactions

Polymerase chain reactions (PCRs) were performed using the “megaprimer” method (Landt *et al.*, 1990; Sarker and Sommer, 1990). All primers were synthesized on an Applied Biosystems 392 DNA Synthesizer (Max Planck Institute, Cologne) and were desalted and deprotected and used directly for PCR. 200 ng of plasmid DNA template was transferred to a sterile 0.5 ml PCR tube to which the following reagents were then added: 5 µl 10 x PCR buffer (670 mM Tris-HCl (pH 8.8), 67 mM MgCl₂, 1.7 mg.ml⁻¹ BSA, 166 mM (NH₄)₂SO₄, 2 µl 25 mM MgCl₂), 1 µl 10 mM dNTPs (10 mM with respect to dATP, dCTP, dGTP and dTTP), 0.5 µl of each oligonucleotide primers (240 µM), 0.5 µl Taq polymerase (40 units/µl, Promega) and the reaction volume diluted to 50 µl with DEPC-treated dH₂O. All reagents were mixed thoroughly, layered with two drops of mineral oil (Sigma) to reduce evaporation and placed in a thermal cycler (Perkin-Elmer 2400, Cetus, USA). The thermal cycler was programmed to denature the sample for 4 min at 94 °C then complete 30 cycles of the 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing) and 74 °C for 1 min (extension). At the end of the last cycle, the sample was heated at 74 °C for a further 10 min to ensure full extension of the product. The PCR reactions were stored at -20 °C. PCR products were analysed by gel electrophoresis as described in 2.9.1.

2.16 Northern Blotting

RNA was separated on a denaturing agarose gel as described in 2.9.3. A wick of Whatman 3 MM paper was placed onto a support and soaked in 20 x SSC (3 M NaCl, 0.3 mM *tri*-sodium citrate). The wick was also dipped into a reservoir of 20 x SSC. The gel was placed on top of the wick with the well side of the gel face down and care taken to remove air bubbles between the wick and the gel. A piece of nylon membrane (Hybond-N, Amersham) was cut to size, rinsed in sterile dH₂O and placed on top of the gel. Any air bubbles between the nylon and the gel were removed using a plastic pipette. Two pieces of Whatman 3 MM paper cut to the size of the gel were rinsed in sterile dH₂O and placed on top of the nylon membrane. The area of the wick within a few mm of the gel was then covered with cling film to prevent short circuiting and a large quantity of paper towels along with a 500 g weight placed on top. The blot was left overnight and then disassembled. The gel and filter were examined under UV light to assess the transfer of RNA from the gel to the nylon. After successful transfer, the filter was briefly rinsed in sterile dH₂O and the RNA fixed using a UV-crosslinker (CL-100 UV-crosslinker, UVP, UK) to deliver 120,000 $\mu\text{Joules.cm}^{-2}$.

2.17 Radiolabelling of DNA

2.17.1 Preparation of DNA to be Labelled

The DNA sequences to be labelled were cut from their host plasmid using the appropriate restriction enzyme(s) and purified as described in 2.10. The amount of the DNA was estimated by running an aliquot of the probe on an agarose gel as described in 2.9.1 and comparing it to the known amounts of DNA loaded onto the same gel.

2.17.2 Random Priming of dsDNA

DNA was labelled with [α - 32 P]dCTP using the Rediprime DNA labelling kit supplied by Amersham and was used in accordance with the manufacturer's instructions. Approximately 25 - 50 ng of DNA, prepared as described in 2.17.1, was diluted to a volume of 45 μ l with sterile dH₂O in a 1.5 ml Eppendorf[®] tube. The DNA was denatured by heating the sample to 95 °C in a boiling water bath for 5 min and briefly microcentrifuged. The denatured DNA was added to the labelling mix (dATP, dGTP, dTTP, exonuclease free Klenow enzyme and random nonamer primers) and the mixture reconstituted by gently flicking the tube until the blue colour of the labelling mix was evenly distributed. After a brief microcentrifugation, 50 μ Ci (3000 Ci.mmol⁻¹) of [α - 32 P] dCTP was added to the reaction mixture and the tube incubated at 37 °C for 15 min. After this, the reaction was stopped by the addition of 5 μ l of 0.5 M EDTA and a further 45 μ l of sterile dH₂O. For use in hybridisation analysis, DNA was denatured by heating to 95 - 100 °C for 5 min and transferred to ice until required. This was carried out after assessing the level of incorporation and removal of unincorporated radionucleotides.

2.17.3 Separation of Labelled DNA from Unincorporated Radionucleotides using Spin Columns

A sterile 1 ml syringe barrel was plugged with siliconised glass wool and filled with Sephadex TESH previously made by autoclaving Sephadex G50 medium (Pharmacia, Milton-Keynes, UK) in a 20-fold volume of TESH (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl, 0.05% (w/v) SDS). The column was spun in a 15 ml Corex[®] tube at 700 g for 4 min at room temperature and the process repeated until the column volume was 1 ml. 100 μ l of TESH was loaded onto the top of the column and centrifuged as before. The TESH wash was repeated once more before the solution of labelled DNA and un-incorporated nucleotides were loaded onto the column. A clean screw-top Eppendorf[®] tube was placed under the column to collect the eluate and the

column centrifuged as before. The DNA was stored at 4 °C until use and the column discarded.

2.17.4 Measurement of the Incorporation of Radionucleotides into DNA Probes

The level of incorporation of radioactivity into DNA probes was estimated by removing 2 µl aliquots of the probe solution before and after centrifugation through the sephadex column described in 2.17.3. The 2 µl samples were placed into screw-cap Eppendorf® tubes containing 0.5 ml dH₂O. These were then inserted into separate scintillation vials and the radioactivity counted (LKB 1209 rackbeta scintillation counter). The percentage of incorporation could then be determined by comparing the counts of the two radiolabelled sample aliquots before and after the centrifugation step: (counts after centrifugation/counts before centrifugation) x 100. The specific activity of the labelled probe (cpm incorporated per µg DNA) could then be calculated, given that the amount of radiolabelled deoxy-nucleotide, template DNA and percentage incorporation of the radiolabel were known. A typical Rediprime reaction with an incorporation of 60 % would yield a specific activity of 1.7×10^9 cpm.µg⁻¹.

2.18 Hybridisation Analysis of Northern Blots

2.18.1 Hybridisation Analysis using Homologous Probes

Northern blots were analysed using the method of Church and Gilbert (1984). Nylon filters prepared as in 2.16 were pre-hybridised at 55 °C in a hybridisation tube (Technique, Cambridge, UK) in a hybridisation oven for 1 - 2 h. Enough pre-hybridisation solution (0.5 M Na₂HPO₄ (pH 7.2), 7 % (w/v) SDS, 10 mg.ml⁻¹ BSA) was used to cover the membrane filter. The radiolabelled cDNA probe was denatured (2.17.2) and added to the pre-hybridisation solution. Hybridisation was then carried out at 55 °C for 16 h.

2.18.2 Washing of Northern Blots

After hybridisation, filters were washed initially in approximately 200 ml 2 x SSC, 1 % (w/v) SDS at 55 °C in a hybridisation tube. The filter was washed at increasing stringency depending on the amount of radioactivity bound to the membrane. Each wash was for 10 min unless otherwise stated. Filters were rinsed in 2 x SSC before autoradiography.

2.18.3 Autoradiography

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

2.18.4 Stripping Filters of Bound Radiolabelled Probes and Blocking Agents

After hybridisation and autoradiography, bound probe and blocking agents were removed by pouring a boiling solution of 0.1% (w/v) SDS over the filter. The solution was allowed to cool to room temperature, and the process repeated a further two times. After this, the filter was wrapped in cling film and autoradiographed overnight to verify that the radiolabelled probe had been removed. The presence of RNA on the filter after stripping could then be detected as described in 2.18.5.

2.18.5 Staining of Filter-Bound RNA with Methylene Blue

This procedure was carried out as described by Sambrook *et al.* (1989). The filter containing the bound RNA was placed in 0.04 % (w/v) Methylene Blue, 0.5 M NaOAc (pH 5.2) for 5 - 10 min. The filter was then washed in DEPC-treated dH₂O until the RNA could be visualised as blue bands on a white background. The stain was removed by washing in 20 % (v/v) acetic acid until the bands were no longer visible.

2.19 Protoplast Isolation

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

2.19.1 Isolation of Light-Grown *Arabidopsis* Cell Culture Protoplasts

This method is a modified version of that obtained from Dr Bernd Weisshaar (Max Planck Institute, Cologne) and was used to prepare protoplasts from both the *At-glw* and the *hy4* cell cultures. The *At-glw* and *hy4* cell cultures were both used 3 days after subculture unless otherwise stated. 50 ml of cells were aseptically transferred to a 50 ml centrifuge tube, pelleted at 150 g for 5 min and the supernatant discarded. An additional step was included for the *hy4* culture in which cell clumps were broken down using a sterile scalpel blade under aseptic conditions. Cells were washed in 40 ml 0.24 M CaCl₂ and centrifuged as before. The supernatant was decanted, the cells resuspended in 70 ml digestion media (1 % (w/v) cellulase R10, 0.25 % (w/v) macerozyme R10, 0.24 M CaCl₂ and heat treated at 55 °C unless otherwise stated) and then poured into a 13.5 cm diameter petri dish. The cells were then incubated for 16 h in the dark at room temperature with constant shaking (30 rpm). After digestion, cell debris was removed by filtering the protoplasts through one layer of muslin and a 100 µm steel sieve (Sigma) into 2 x 50 ml centrifuge tubes. Protoplasts were centrifuged as above, washed in 25 ml of 0.24 M CaCl₂ and centrifuged once more. The supernatant was decanted and the final pellet resuspended in 10 ml culture media (0.4 M sucrose, 1 x Gamborg's B5 salts (Sigma) (pH 5.8)) and transferred to a 12 ml centrifuge tube. Viable protoplasts were purified and concentrated by centrifuging as above for 10 min. Living protoplasts float as a band on top of the sucrose and were transferred to a clean 12 ml centrifuge tube with a 1 ml pipette and this purification step repeated. Protoplast viability and number were then determined before use (2.19.3 and 2.19.4).

2.19.2 Isolation of At-7 Cell Culture Protoplasts

The At-7 cell culture was used 6 days after subculture. 40 ml was aseptically transferred to a 50 ml centrifuge tube. The cells were pelleted at 150 g for 5 min and the supernatant discarded. The cell pellet was then resuspended in 40 ml digestion media (1.7 % (w/v) cellulase R10, 0.1 % (w/v) macerozyme, 0.24 M CaCl₂), gently resuspended and 20 ml transferred to 2 x 13.5 cm diameter petri dishes containing a further 10 ml digestion media. The cells were then incubated for 16 h in the dark at room temperature with constant shaking (30 rpm). Cell debris was removed by filtering the protoplasts through 100 µm and 61 µm nylon sieves into 2 x 50 ml centrifuge tubes. The protoplasts were then washed, purified and concentrated as described in 2.19.1.

2.19.3 Determination of Protoplast Viability

Protoplast viability was determined using fluorescein diacetate (FDA). A stock solution of FDA (5 mg.ml⁻¹) was prepared in acetone and stored in the dark at 4 °C. Two drops of the FDA solution were added to 20 ml of the appropriate protoplast culture medium and mixed. 200 µl of this solution was mixed with an equal volume of the protoplast suspension and incubated at room temperature for 2 min. Protoplast viability was then determined using a fluorescence microscope (using blue illumination). Living protoplasts stained green whereas non-viable protoplasts stained red or remained unstained. The percentage viability in a random field of view could then be determined: (number of green fluorescing protoplasts/total number of protoplasts) x 100.

2.19.4 Counting Protoplasts

Protoplasts were counted using a haemocytometer. A sample of the protoplast suspension (of a known volume) was introduced beneath the cover-slip, to fill the counting area of the haemocytometer. Using a microscope, the number of protoplasts enclosing the triple-lined square (enclosing 16 smaller squares) were counted (n), including those touching the top and left edges but not the bottom or right edges. The

number of protoplasts per ml was calculated as $5n \times 10^3$. Protoplasts were then diluted to the required concentration in the appropriate buffer.

2.20 Transient Gene Expression in Protoplasts

This is a modified version of the method obtained from Dr Bernd Weisshaar (Max Planck Institute, Cologne) and was used with all three cell cultures. Protoplasts were diluted to a concentration of 1×10^6 per ml with the appropriate buffer. 200 μ l of the protoplast suspension was transferred to a round bottom 12 ml centrifuge tube, 20 μ g of the appropriate plasmid DNA added and gently mixed. 200 μ l of polyethylene glycol (PEG) solution (25 % (w/v) PEG 6,000 (Serva) 100 mM $\text{Ca}(\text{NO}_3)_2$ (Sigma), 45 mM mannitol, pH 9.0) was added, gently mixed and incubated at room temperature for 15 min. After this period, 5 ml wash solution (275 mM $\text{Ca}(\text{NO}_3)_2$) was added and the tube centrifuged at 150 g for 5 min. The supernatant was decanted and the protoplasts gently resuspended in 6 ml culture media (0.4 M sucrose, 1 x Gamborg's B5 salts (Sigma) (pH 5.8)) or an alternative culture medium containing (a) sugar(s) other than sucrose (see Chapter 6). Transfections involving the same plasmid were pooled together and mixed before each experiment in order to avoid variation in transfection efficiencies between samples. Protoplasts were separated into 3 ml aliquots in 12 ml UV-transmitting plastic centrifuge tubes and given the specified light (or dark) treatment.

2.21 Harvesting of Protoplasts

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

2.22 Measurement of GUS Activity

Levels of β -glucuronidase (GUS) activity were measured by the method of Jefferson (1987) using 4-methyl umbelliferyl glucuronide (MUG) as substrate (Biogene, Bolnhurst, UK). Harvested protoplasts were thawed on ice before resuspending in 200 μ l of GUS extraction buffer (50 mM NaPO_4 (pH 7.0), 10 mM β -mercaptoethanol, 10 mM EDTA, 0.1 % (w/v) sodium lauryl sarcosine, 0.1 % (w/v) Triton X-100). The protoplast suspension was vortexed thoroughly, centrifuged at 10,000 g for 5 min and returned to ice. 100 μ l of the soluble fraction was transferred to a clean 1.5 ml Eppendorf[®] tube containing 100 μ l of 2 mM MUG (dissolved in GUS extraction buffer) and inverted to mix. The remaining 100 μ l of sample was kept for protein determination (2.22). As a blank, a tube was prepared containing equal volumes (100 μ l) of GUS extraction buffer and substrate. Tubes were then incubated at 37 °C in a water bath. At intervals of 20, 40 and 60 min, the reaction was stopped by removing 40 μ l from each tube to a 2 ml Eppendorf[®] tube containing 1.96 ml of 0.2 M Na_2CO_3 . These tubes were then mixed and the relative concentration of 4-methylumbelliferone (4-MU) production quantified using a spectrofluorimeter (Perkin Elmer LS5), excitation at 365 nm and emission at 455 nm. The 20, 40 and 60 min fluorescence values for the blank were then subtracted from the values for each sample to normalise against substrate decomposition at 37 °C. GUS activity was calculated by calibrating the fluorimeter with solutions of 4-MU (Sigma). A 100 mM stock solution of 4-MU in 50 % (v/v) ethanol was prepared and serially diluted 1/10 in GUS extraction buffer : Na_2CO_3 (1 : 50) to obtain a series of standards (1, 10, 100, 1000 nM). The 4-MU concentration in each sample was calculated from a standard curve of relative fluorescence against 4-MU concentration (nM). GUS activity was then calculated as pmoles MU produced per min and normalised per mg protein as determined in 2.23. In transfections with protoplasts prepared from the At-7 cell culture, GUS activity was normalised using a ubiquitin promoter-driven luciferase gene as an internal standard. Where luciferase (LUC) activity was to be determined, GUS extraction buffer was

replaced with LUC extraction buffer (100 mM potassium phosphate pH 7.5, 1 mM DTT). Luciferase activity was determined in 10 μ l of the protoplast extract by addition of 100 μ l LUC assay buffer (20 mM Tricine, 1.07 mM $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$, 2.67 mM MgSO_4 , 0.1 mM EDTA, 33.3 mM DDT, 270 μ M CoA, 470 μ M luciferin, 530 μ M ATP, pH 7.8) and subsequent determination of the photons emitted over 10s in a luminometer (Lumat LB9501, Berthold, Germany).

2.23 Measurement of Protein

Protein levels were measured using the method of Bradford (1976) using BSA (Sigma) as standard. Bradford reagent was prepared by dissolving 100 mg of Coomassie brilliant blue G250 in 50 ml 95 % (v/v) ethanol and 100 ml 85 % (w/v) phosphoric acid. This was diluted to 1:1 with dH_2O and filtered through Whatman filter paper before use.

The protein sample of interest (5 - 10 μ l) was diluted to 100 μ l with dH_2O in a 1 ml cuvette. From a $1\text{mg}\cdot\text{ml}^{-1}$ stock solution of BSA, 2, 4, 8, 16 and 32 μ g were prepared by diluting to 100 μ l with dH_2O in a 1 ml cuvette. 1 ml Bradford's reagent was added to each cuvette, mixed by inversion and incubated at room temperature for 15 min. Levels of protein were determined by measuring the OD at 595 nm in a spectrophotometer. The protein standards were used to construct a standard curve of absorbance at 595 nm against amount of protein (μ g). The amount of protein (μ g) in each sample was then calculated.

CHAPTER 3: DEVELOPMENT OF A PROTOPLAST TRANSIENT EXPRESSION SYSTEM IN *ARABIDOPSIS*

3.1 INTRODUCTION

In order to define the light responsive *cis*-acting elements concerned with the transcriptional activation of *AtCHS*, we developed a UV/blue light inducible transient expression system. This system involved transfection of chimaeric *AtCHS* promoter constructs into *Arabidopsis* cell culture protoplasts. The reporter enzyme β -glucuronidase (GUS) was used to monitor light-responsive promoter activity. Development of the system involved defining and optimising procedures for producing protoplasts from the *Arabidopsis* cell culture, transfection of chimaeric *AtCHS* promoter constructs into the protoplasts and incubations under defined illumination conditions. This chapter outlines the experiments performed to develop and optimise an efficient protoplast transient expression system which was subsequently used to functionally analyse the light-responsive *AtCHS* promoter.

3.1.1 A Light-Responsive System to Study *CHS* Promoter Activity

Initial research with Parsley (*Petroselinum crispum*) showed that protoplasts retain similar responsiveness to UV light as suspension-cultured cells which had been previously grown in darkness (Dangl *et al.*, 1987). This allowed chimaeric gene constructs to be introduced into parsley protoplasts in order to analyse the effects of promoter manipulations on the light-dependent expression of a reporter gene, such as the *uidA* gene encoding β -glucuronidase (GUS) (Jefferson *et al.*, 1986). In this way, it is possible to determine the activity of the *CHS* promoter simply by measuring specific GUS activity. Previous studies in parsley have defined promoter elements and transcription factors involved in the regulation of *CHS* gene expression by UV-

containing white light. Four *cis*-acting elements were identified in the *PcCHS* promoter that were bound by proteins following illumination with UV-containing white light (Schulze-Lefert *et al.*, 1989a; Schultz-Lefert *et al.*, 1989b). Two of these elements (ACE^{PcCHS} and MRI^{PcCHS}) were shown to bind bZIP and MYB classes of transcription factors (Feldbrügge *et al.*, 1994; Feldbrügge *et al.*, 1997).

Although parsley, in particular, has proven to be an excellent system for studying the basic aspects of the control of *CHS* promoter activity, it is not an ideal system in which to further dissect the cellular and molecular mechanisms involved in the light regulation of *CHS*. This is simply because it is not well suited to a genetic approach. The availability of mutants would enable researchers to define the functions of specific members of the families of transcription factors that interact with the *CHS* promoter.

It was therefore decided to develop a system to study *CHS* promoter activity in *Arabidopsis thaliana*. Feinbaum *et al.* (1991) reported an initial study of the promoter elements of the *Arabidopsis thaliana CHS* (*AtCHS*) gene which may be involved in light regulation. However, although this study revealed those wavelengths of light important for *AtCHS* expression in transgenic *Arabidopsis* plants, several questions remained unresolved. In order to answer some of those questions, we have used a photomixotrophically grown cell suspension culture (*At-glw*) and developed a homologous protoplast transient expression system to define the elements of the *AtCHS* promoter involved with induction by UV-B and UV-A/blue light.

3.1.2 Production of Protoplasts from Suspension-Cultured *Arabidopsis* Cells

Previous work (Schulze-Lefert *et al.*, 1989a/b; Dangl *et al.*, 1987) had shown that protoplasts isolated from cultured parsley cells responded to UV-containing white light by activation of the endogenous *PcCHS* gene. Furthermore, when transfected with

appropriate constructs, UV-containing white light induced expression of a reporter gene under the control of the *PcCHS* promoter.

A method similar to that described for parsley (Dangl *et al.*, 1987) was used to prepare *Arabidopsis* cell culture protoplasts. Protoplasts can be produced from light-grown At-glw cells by treatment with the cell wall degrading enzymes, cellulase and macerozyme. Enzymic digestion of cell tissue was complete after a 16 h incubation period at room temperature. Protoplasts prepared in this manner were routinely found to be of high viability (almost 100%) when stained with fluorescein diacetate (FDA), even 24 h after isolation (see Figure 3.1).

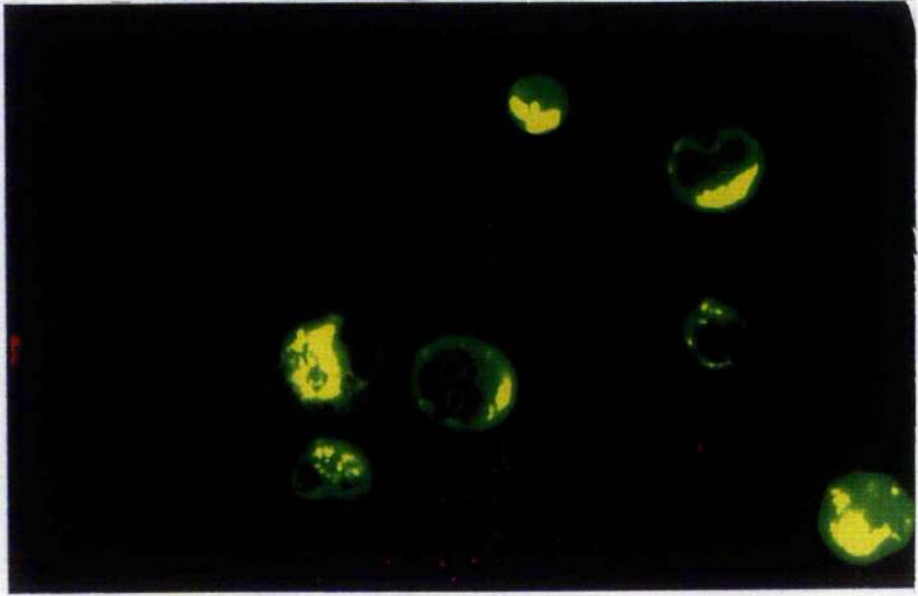
Previous northern analysis from our laboratory has revealed that *AtCHS* transcript levels are very low in At-glw protoplasts illuminated with a low fluence rate of white light and increase following transfer to a 5-fold higher fluence rate (Christie, 1996). Transfer of protoplasts to UV-B or UV-A/blue also induces *CHS* transcript accumulation (Hartmann *et al.*, 1998). In addition, it was shown that irradiation with red light did not induce *CHS* gene activity (Christie, 1996). Thus the photoregulation of *CHS* expression in protoplasts is very similar, if not identical, to that observed for the At-glw cell culture (Christie and Jenkins, 1996) and for intact mature leaf tissue (Jackson *et al.*, 1995; Funglevand *et al.*, 1996; Kubasek *et al.*, 1992). These results clearly demonstrate that protoplasts derived from the At-glw cell culture provide a suitable homologous system in which to study *AtCHS* promoter activity.

3.1.3 Initial Transient Expression in At-glw Cell Culture Protoplasts

Transient expression of genes in plant protoplasts has been shown to provide a rapid method for analysing essential *cis*-acting elements regulating transcription. In fact, transient gene expression assays have been established in a number of plant systems,

Figure 3.1 Photograph of FDA stained At-glw protoplasts

The photograph shows protoplasts produced from At-glw cell culture three days after subculturing. Protoplasts are stained for viability with FDA and viewed under a fluorescence microscope at a magnification of x 200. Live protoplasts stain green whereas non-viable protoplasts stain red.



including *Arabidopsis* (Damm *et al.*, 1989; Axelos *et al.*, 1992; Abel and Theologis, 1993; Doelling and Pikaard, 1993). Initial studies in our laboratory investigated the use of electroporation and PEG-mediated transfection of DNA into cell culture protoplasts (Christie, 1996). Firstly, electroporation of plasmids encoding a reporter gene driven by the constitutively expressed cauliflower mosaic virus 35S promoter was studied. Despite electroporation being performed over a range of capacitances and voltages, this process was largely unsuccessful and very little reporter activity was detected in the protoplasts (data not shown). PEG-mediated transfection was then investigated using a method similar to that described for parsley (Lipphardt *et al.*, 1988). In these experiments reporter gene constructs were found to be highly expressed in protoplasts isolated from the cell culture. In addition, it was shown that the *AtCHS* promoter could drive reporter gene expression in a light regulated manner in these protoplasts following PEG-mediated transfection. These results indicated that PEG transfection could be used to introduce chimaeric gene constructs into protoplasts and one could subsequently demonstrate high levels of transgene expression.

3.2 TRANSIENT EXPRESSION IN *At-glw* PROTOPLASTS

3.2.1 Factors Affecting Reporter Gene Activity

Before beginning the analysis of *CHS* promoter activity, we performed experiments designed to identify conditions under which transient *CHS-GUS* expression levels could be improved in this *Arabidopsis* protoplast system. In similar parsley systems, protoplasts are routinely isolated from suspension culture cells 5 days after subculture (Lipphardt *et al.*, 1988; Block *et al.*, 1990). In contrast, in previous experiments with the *At-glw* cell culture we had used cells 3 days after subculture (the point at which the cells enter the exponential growth phase) to produce protoplasts. We decided therefore to examine whether the age of the cells used affected *CHS* promoter activity. *Arabidopsis* protoplasts were isolated from either 3 or 5 day old suspension cultures

and transfected with *CHS-GUS* using PEG. After transfection, the protoplasts were incubated in the dark or under a high fluence rate of white light for 16 h. The protoplasts were then harvested and assayed for GUS activity. The results are shown in Figure 3.2.

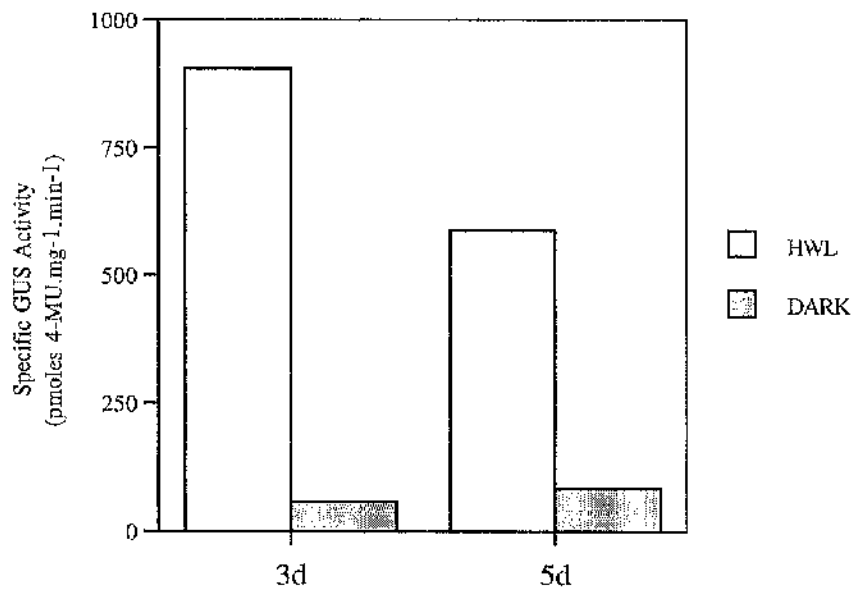
These results clearly show that protoplasts isolated from both 3 and 5 day old *At-glw* cells support a high level of light-induced *CHS-GUS* expression. Further, *CHS-GUS* expression was no greater in protoplasts isolated from 5 day old cells. Indeed, it appears that GUS activity was slightly lower in these protoplasts. Based on these results, all subsequent experiments were performed using protoplasts produced from cells 3 days after subculture. A notable point from this experiment was that induction levels vary somewhat between separate experiments. This may have been due to differences in protoplast quality, in terms of competence in supporting transient expression based on the observation of poor levels of activity from the CaMV 35S promoter, or perhaps in transfection efficiency. This variation indicated that further refinement of the transient expression system was required before beginning analysis of the *CHS* promoter.

3.2.2 The Effect of Different Cellulase Preparations on Protoplast Production

One possible explanation for the variation between separate experiments observed thus far was poor quality protoplasts. Previous experiments had revealed that although FDA staining may indicate protoplasts have high viability, they do not always support high levels of transient *CHS-GUS* expression (data not shown). In addition, Dr. Bernd Weisshaar at the Max Planck Institute for Plant Breeding in Cologne had observed considerable differences between different lots of cellulase in terms of success in protoplasting. One of the possible explanations for the production of poor quality

Figure 3.2 Effects of cell age on *AtCHS* promoter activity

Protoplasts isolated from 3 day (3d) or 5 day (5d) old *At-glw* cells were transfected with 20 μg of pAtCHS -1972 DNA and transferred to darkness or 100 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light for 20 h. After incubation, protoplasts were harvested and GUS activity determined. The experiment was performed three times and values are shown of a representative experiment.



protoplasts in our system was the use of an unsuitable batch of cellulase. We decided therefore to examine whether preparation of protoplasts using different batches of cellulase affected the level of transient *AtCHS-GUS* expression.

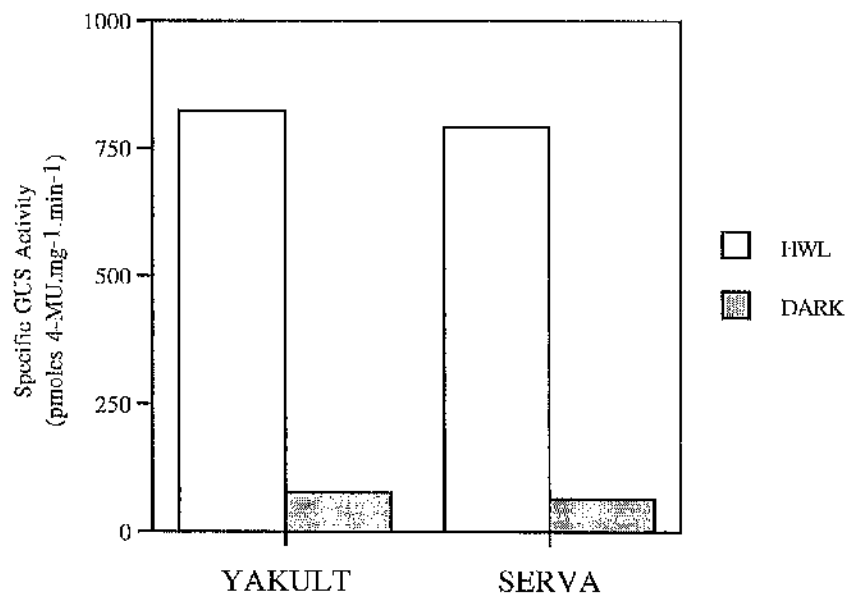
Two separate batches of cellulase R-10 (A and B) from different suppliers were used to produce protoplasts for transient expression in order to determine which batch, if any, produced protoplasts capable of supporting high levels of transient *AtCHS-GUS* expression. Batch A was Cellulase R-10 from Serva (Heidelberg, Germany), batch number 04530, which was known to have been successfully used to produce protoplasts for transient expression from a very similar cell-suspension culture in the laboratory of Dr. Bernd Weisshaar in Cologne. Batch B was Cellulase R-10 from Yakult Pharmaceuticals Ltd. (Tokyo, Japan), batch number 12796, and this batch had been used to produce protoplasts for each of the transient expression experiments described above.

Two preparations of protoplasts were produced from light-grown suspension-cultured *Arabidopsis* cells by treatment with batches A and B of cellulase and macerozyme. Enzymic digestion of cell tissue was complete after a 16 h incubation period at room temperature and the protoplasts were then transfected with *AtCHS-GUS* using PEG. After transfection, the protoplasts were incubated in the dark or under a high fluence rate of white light for 16 h. The protoplasts were then harvested and assayed for GUS activity.

The results, shown in Figure 3.3, indicate that protoplasts produced from both batches A and B of cellulase were capable of supporting high levels of light induced *AtCHS-GUS* expression. It appears that there are no apparent differences in levels of light-induced reporter gene activity in protoplasts prepared from cellulase batches A and B. It subsequently became apparent that induction levels vary somewhat between separate experiments for protoplasts prepared with either batch A or batch B of cellulase.

Figure 3.3 Effect of different cellulase preparations on *AtCHS* promoter activity

Two different protoplast suspensions were isolated from *At-glw* cells using either a 1 % Yakult cellulase: 0.25 % macerozyme solution (YAKULT) or a 1 % Serva cellulase: 0.25 % macerozyme solution (SERVA). Protoplasts were transfected with 20 μg of pAtCHS -1972 DNA and transferred to darkness or 100 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light for 20 h. After incubation, protoplasts were harvested and GUS activity determined. The experiment was performed three times and values of a representative experiment are shown.



3.2.3 The Effect of Enzyme Concentration on Protoplast Preparation

The concentrations of cellulase and macerozyme used to prepare protoplasts from the *At-glw* cell culture are equivalent to those required to isolate protoplasts from *Arabidopsis* leaf tissue (Damm *et al.*, 1989; Abel and Theologis, 1994). Lower concentrations are used to generate protoplasts from parsley cell cultures (Dangl *et al.*, 1987). This prompted us to examine whether a lower concentration of cellulase and macerozyme could be used to isolate protoplasts from the *At-glw* cell culture and whether these protoplasts support high levels of *AtCHS-GUS* activity without the high degree of variation observed between separate experiments.

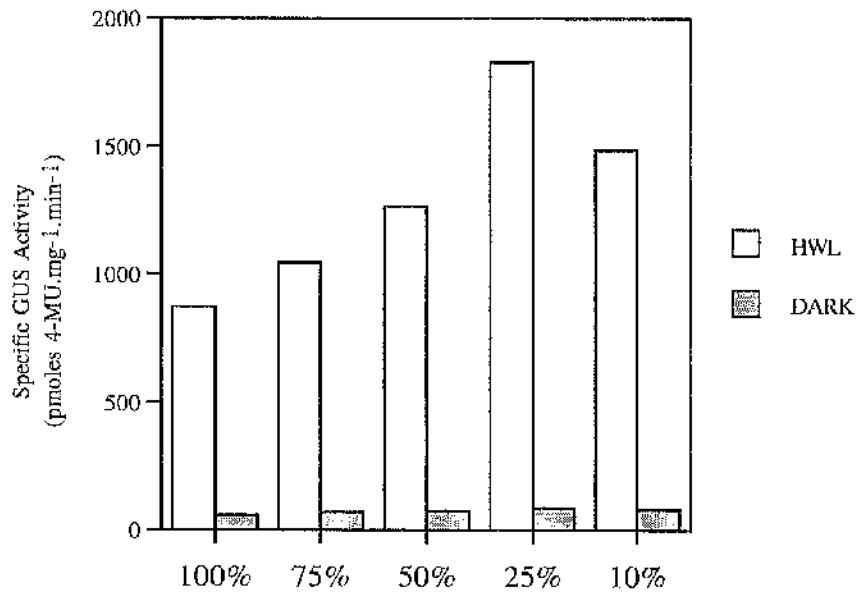
Five batches of protoplasts were prepared with either the standard enzyme solution (containing 1% cellulase and 0.25% macerozyme) or dilutions of this standard enzyme solution and used for transient gene expression studies. Although protoplast yield decreased at lower enzyme concentrations, viability was unaffected (data not shown).

We observed that light-induced *AtCHS* promoter activity was significantly higher in protoplasts obtained from solutions containing low concentrations of cellulase and macerozyme (see Figure 3.4). Indeed, maximal GUS activity was observed in extracts from protoplasts prepared in a solution of 0.25% cellulase and 0.06% macerozyme, similar to concentrations used to isolate protoplasts from parsley cells (Dangl *et al.*, 1987). These findings appear to indicate that an unnecessarily high concentration of cellulase and/or macerozyme can reduce transgene expression in plant protoplasts or perhaps reduce the transfection efficiency of the protoplasts or both.

This experiment was repeated four times (data not shown) and although we observed maximal GUS activity in extracts from protoplasts prepared in a solution of 0.25% cellulase and 0.06% macerozyme in each case, we also noted that induction levels still varied somewhat between experiments. As a result of this, we decided to undertake

**Figure 3.4 Effect of cell wall degrading enzyme concentration on
AtCHS promoter activity**

Five different protoplast suspensions were isolated from *At-glw* cells using either the standard enzyme solution (100 %) at a concentration of 1 % cellulase: 0.25 % macerozyme, 3/4 dilution of the standard enzyme solution in 0.24 M CaCl₂ (75 %), 1/2 dilution (50 %), 1/4 dilution (25 %) or a 1/10 dilution (10%). Protoplasts were transfected with 20 µg of pAtCHS -1972 DNA and transferred to darkness or 100 µmol.m⁻².s⁻¹ white light for 20 h. After incubation, protoplasts were harvested and GUS activity determined. The experiment was performed three times and values of a representative experiment are shown.



further experiments to refine our protoplast preparation procedure before beginning analysis of the *AtCHS* promoter.

3.2.4 The Effect of Enzyme Solution Heat Treatment on Protoplast Preparation

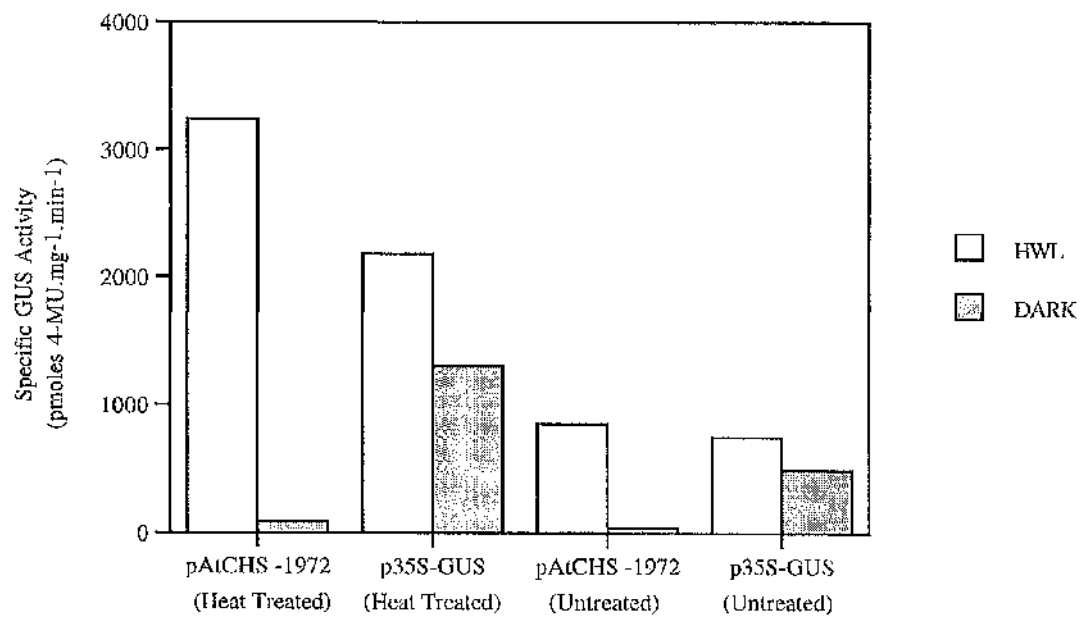
It came to our attention that Dr. J. Sheen and co-workers at the Massachusetts General Hospital in Boston were performing transient assays using *Arabidopsis* mesophyll protoplasts. Personal communication with Dr. Sheen revealed that these researchers heated all cellulase/macerozyme solutions to 50-55 °C for 10 minutes and allow them to cool to room temperature before use. It is thought that such a heat treatment can help to inactivate harmful proteases as well as accelerating enzyme solubilization. We therefore decided to investigate the effect of such a heat treatment on protoplast preparation and levels of reporter gene expression in our transient assay system.

In order to do this, two batches of protoplasts were prepared using either the standard untreated enzyme solution (containing 1% cellulase and 0.25% macerozyme) or the same solution which had been heated at 55 °C for 10 minutes (and allowed to cool to room temperature) before being used for transient gene expression studies. It appeared that when the enzyme solution was heat treated, the protoplast yield was slightly increased and FDA staining showed that viability remained unaffected (data not shown). The results of transient expression assays using these protoplasts are shown in Figure 3.5.

This experiment clearly shows that heat treatment of the enzyme solution prior to incubation with the cells produced protoplasts which supported much higher levels of GUS reporter gene expression from both the light-induced *AtCHS* promoter and the constitutive *CaMV 35S* promoter. It remains unclear as to whether this increased GUS activity is due to the protoplasts prepared using the heat treated enzyme solution

Figure 3.5 Effect of heat treatment of cell wall degrading enzyme solution on *AtCHS* promoter activity

Two different protoplast suspensions were isolated from *At-glw* cells using either a 1 % cellulase: 0.25 % macerozyme solution heated to 55 °C for 10 min (Heat Treated) or an unheated enzyme solution (Untreated). Protoplasts were transfected with either 20 µg of pAtCHS -1972 DNA or 20 µg of p35S-GUS DNA and transferred to darkness or 100 µmol.m⁻².s⁻¹ white light for 20 h. After incubation, protoplasts were harvested and GUS activity determined. The experiment was performed three times and values of a representative experiment are shown.



showing increased transfection efficiencies or supporting higher levels of transient gene expression or both.

When the above experiment was repeated a further three times, it was found that GUS activity in extracts from protoplasts prepared using heat treated enzyme solution showed only a very slight variation between separate experiments (data not shown). This was in contrast to the considerable variation of induction levels observed in separate assays which were performed with protoplasts prepared using untreated enzyme solution. In addition, we also observed that protoplasts produced using the heat treated enzyme solution survived 2-3 days longer than those prepared using untreated enzyme solution when incubated in floating medium (Gamborg's B5 medium (Sigma) containing 400 mM sucrose and 1 mg/l 2,4-D pH 5.7). Protoplasts stayed afloat for 6-7 days when prepared using heat treated enzyme solution as opposed to 4-5 days when prepared using untreated enzyme solution (data not shown). These observations appear to indicate that protoplasts produced from the *At-glw* cell culture using heat treated enzyme solution are of high quality and are suitable for transient expression assays designed to analyse the *Arabidopsis CHS* promoter.

3.2.5 The Effect of Concentration of Heat-Treated Enzyme Solution on Protoplast Preparation

The experiment described in section 3.2.3 (see Figure 3.4) had indicated that the concentration of the enzyme solution was also important for producing protoplasts capable of supporting high levels of transient gene expression. This observation prompted us to investigate how the concentration of cellulase and macerozyme in the enzyme solution affects protoplast preparation and transient gene expression after heat treatment on the enzyme solution. We therefore performed an experiment to optimise the concentration of the enzyme solution prior to heat treatment to prepare protoplasts

which support the highest levels of reporter gene activity and provide consistent values in separate experiments.

The results of this analysis are shown in Figure 3.6. Optimal reporter gene activity was observed in extracts from protoplasts prepared using an enzyme solution of concentration 1.5% cellulase: 0.38% macerozyme and from protoplasts prepared using an enzyme solution of concentration 1.0% cellulase: 0.25% macerozyme prior to heat treatment. This experiment also revealed that concentrations of the enzyme solution below 0.5% cellulase: 0.13% macerozyme produced very low yields of protoplasts when the enzyme solution was heat treated (data not shown).

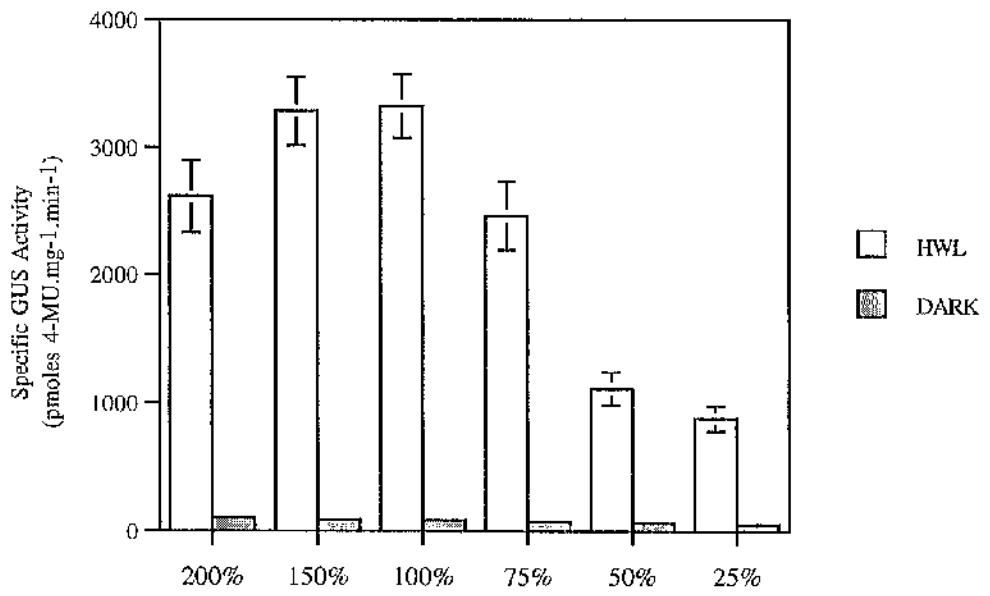
These observations, when compared with the results in section 3.2.3, appear to indicate that some of the activity of the enzyme solution was lost during the heat treatment. As a result, one must use a much higher concentration of enzymes than that indicated in section 3.2.3. It was therefore decided to use an enzyme solution of concentration 1.0% cellulase: 0.25% macerozyme and incubate it at 55 °C for 10 minutes for all subsequent protoplast preparations and transient expression assays.

3.2.6 Optimisation of the Transient Expression Assay

During optimisation of the transient expression assay, an incubation time of between 8 h and 16 h appeared to be optimal for high-fold induction values of the *AtCHS* promoter (see Figure 3.7). Significant activity in the light was observed 6 h (about 35% of maximal activity) after beginning the light treatment, whilst at 2 h and 4 h only background or low activity was detected. At later time points the activity in the light increased only slightly, whilst the dark values increased thereby causing a reduction of the fold induction values. In the case of the 35S promoter, significant activity was already observed after only 2 h and after 4 h about 60% of maximal activity was

Figure 3.6 Effect of concentration of heat treated cell wall degrading enzyme solution on *AtCHS* promoter activity

Six different protoplast suspensions were isolated from *At-giw* cells using different concentrations of the standard enzyme solution (100 %) which is 1 % cellulase: 0.25 % macerozyme solution heated to 55 °C for 10 min. These concentrations are given as percentage values (200 %, 150 %, 75 %, 50 % and 25 %) relating to the standard enzyme concentration (100 %). Protoplasts were transfected with either 20 µg of pAtCHS -1972 DNA or 20 µg of p35S-GUS DNA and transferred to darkness or 100 µmol.m⁻².s⁻¹ white light for 20 h. After incubation, protoplasts were harvested and GUS activity determined. The experiment was performed four times and the mean values are shown. The error bars represent the standard deviation. Where error bars are absent, the SD is too small to accurately represent.



reached. This indicates that the transient expression system also reproduced the lag phase during *AtCHS* gene activation seen at the mRNA level in previous studies (Christie, 1996).

Thus the optimisation of the isolation and assay conditions resulted in the production of protoplasts that were capable of supporting high levels of light-induced reporter gene activity from *AtCHS:GUS* constructs in transient expression assays. In addition, it appeared that when these assays were repeated we could produce consistent values for reporter gene activity in separate experiments. It was now felt that a reliable system had been developed that we could use in transient expression assays to analyse the activity of the *CHS* promoter.

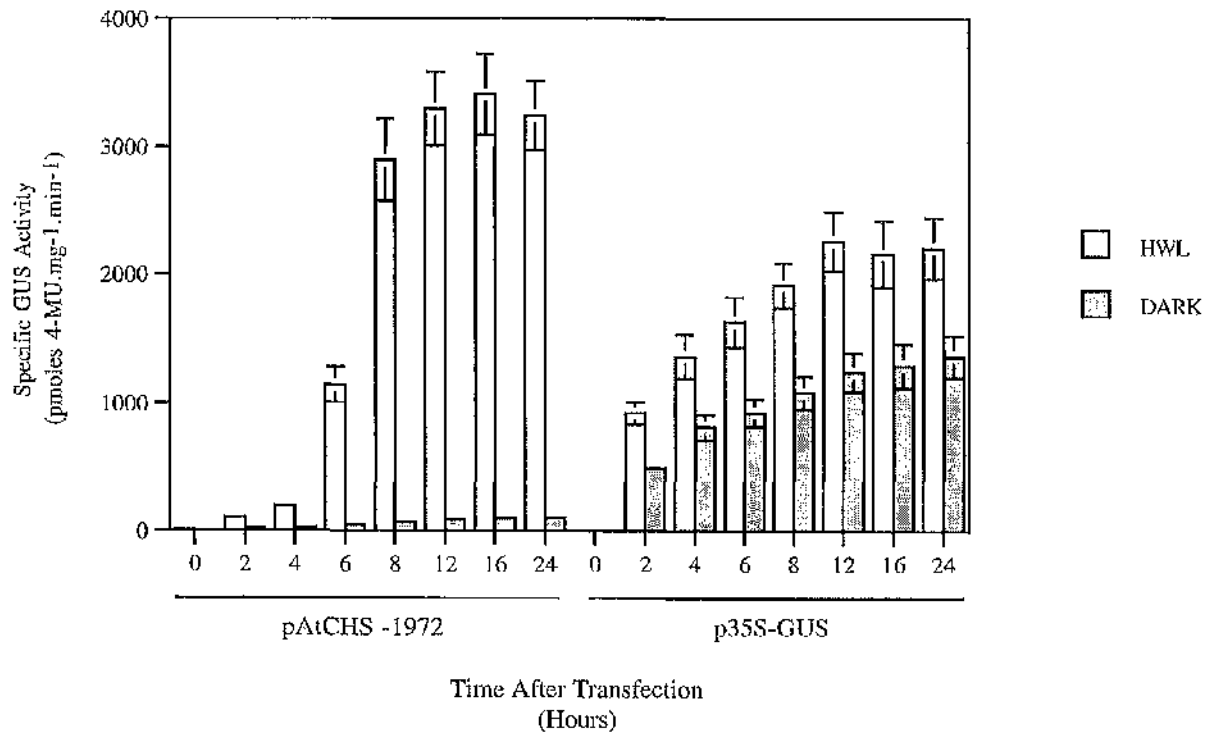
3.2.7 *CHS* Promoter Activity is Light-Regulated in *At-glw* Cell Culture Protoplasts

We decided to perform an experiment to confirm that our optimised transient expression system was indeed responsive to the same light qualities as intact *At-glw* cells and mature leaf tissue and was therefore capable of providing a reliable method of analysis of *CHS* promoter activity. In order to do this, protoplasts isolated from the *At-glw* cell culture were transfected with plasmids containing either the *AtCHS-GUS*, *35S-GUS* or pBT-2 (promoterless *GUS*) reporter gene constructs and transferred to various light treatments for 16 h.

As shown in Figure 3.8, *AtCHS* promoter activity is strongly induced by UV-B and UV-A/blue light. High intensity white light was also very effective whereas no induction was observed in the dark or in response to red light. This result is in agreement with previous northern analyses with *At-glw* protoplasts (Christie, 1996). Furthermore, the lack of induction by red light is consistent with the conclusion that phytochrome has very little effect on *AtCHS* expression in all but the youngest

Figure 3.7 Kinetics of induction of *AtCHS* promoter activity in response to HWL

At-glw protoplasts were transfected with 20 μg of either pAtCHS -1972 DNA or p35S-GUS DNA and transferred to darkness or 100 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light for the times indicated. Protoplasts were harvested and assayed for GUS activity. The times are given in hours. The values shown are the means for four samples. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to accurately present.



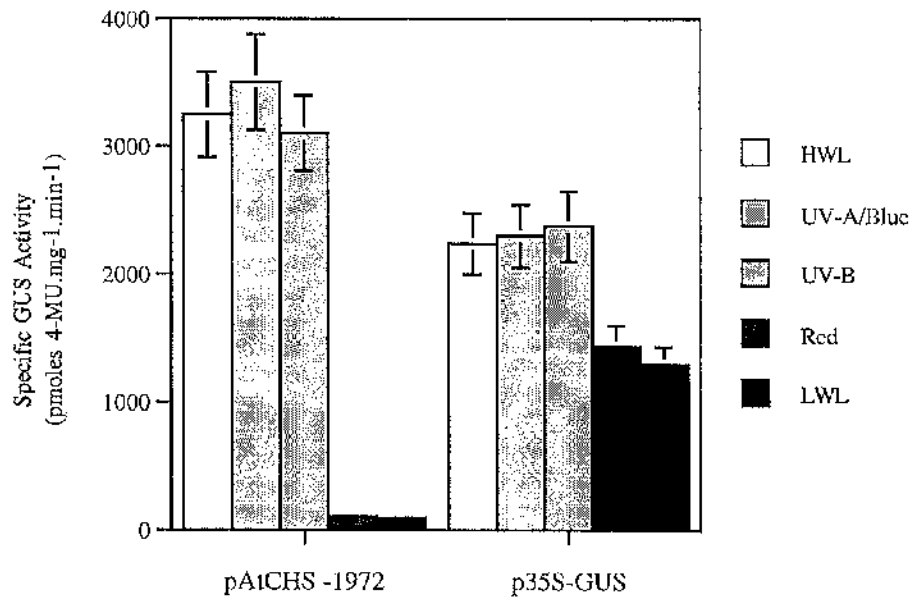
Arabidopsis seedlings (Feinbaum *et al.*, 1991; Kubasek *et al.*, 1992; Kaiser *et al.*, 1995). It would also appear that none of the light treatments had any significant effect on transient *35S-GUS* expression. These observations were in agreement with those published by Christie and Jenkins (1996) confirming the light-responsive nature of the *Arabidopsis* cell culture referred to here as At-glw. Additionally, the consistent values observed between repetitions of this experiment indicated that this transient expression system would provide a suitable means of analysing the *AtCHS* promoter elements required for UV-B and UV-A/blue light induction.

3.3 DISCUSSION

Transient expression analysis in cell culture protoplasts has the advantage over similar systems using leaf protoplasts that the protoplasts prepared from cell culture are much more uniform than those derived from leaf tissue. In addition, *AtCHS* expression is largely confined to the epidermis (Peto *et al.*, 1991) and preparation of large numbers of protoplasts from leaf epidermis is a very labour intensive process. The use of cell culture protoplasts is a great deal more convenient and northern analysis (Christie, 1996), as well as initial transient expression assays (3.2.7), suggest that the protoplasts provide an accurate reflection of the regulation of *AtCHS* transcription in mature plants. In addition, the excellent reproducibility between different experiments with the At-glw protoplasts led us to feel that an internal standard was not required for these transient assays. The development and optimisation of an efficient transient expression system using cell culture protoplasts provides us with a powerful tool to analyse promoter function.

Figure 3.8 Light regulation of *AtCHS* promoter activity in *At-glw* protoplasts

At-glw protoplasts were transfected with 20 μg of either pAtCHS -1972 DNA or p35S-GUS DNA and transferred to HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light), UV-A/Blue ($80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ UV-A/blue light), UV-B ($3 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ UV-B light), Red ($80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ red light) or LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for four samples. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



CHAPTER 4: FUNCTIONAL ANALYSIS OF THE *Arabidopsis* *CHS* PROMOTER

4.1 INTRODUCTION

Having developed an efficient transient expression system, we used it to define the light responsive *cis*-acting elements concerned with the transcriptional activation of *AtCHS*. This was done by preparing various chimaeric *AtCHS* promoter: GUS reporter constructs and analysing their activity in transient expression assays. This analysis showed that a 1972 bp *AtCHS* promoter conferred UV-B and UV-A/blue light induction of GUS activity. Deletion of the promoter to 164 bp resulted in reduced promoter strength but the promoter still showed responsiveness to UV-B and UV-A/blue light. This region contains a light responsive unit (LRU) which consists of an ACGT-containing element (ACE) and a MYB recognition element (MRE). Further deletion abolished promoter activity.

4.1.1 Deletion Analysis of the *AtCHS* Promoter in *At-glw* Protoplasts

In order to identify the functional elements within the *AtCHS* promoter, the effects of 5' deletions of the promoter fused to the GUS reporter gene were assayed by transient expression in *At-glw* protoplasts. The 5' end of the promoter is at position -1972 relative to the transcriptional start as mapped by Feinbaum *et al.* (1991). Deletion constructs were derived from the full-length promoter construct by using appropriate restriction sites in the promoter or via PCR derived fragments by researchers in Dr. Bernd Weisshaar's laboratory in Cologne. All of these plasmid constructions were verified by sequencing to exclude the introduction of mutations during PCR or at the

cloning sites. Figure 4.1 shows the *AtCHS* promoter in diagrammatic form and indicates the positions of the deletions introduced.

The deletion series of constructs was assayed by transfection into At-glw protoplasts under different conditions of illumination: darkness, high intensity white light, UV-A/blue light and UV-B light. The result of this experiment is shown in Figure 4.2. None of the *AtCHS* promoter deletions was active in protoplasts incubated in darkness. Deletion of sequences between -1972 and -668 showed no significant effect on promoter activity under any of the light conditions assayed. However, deletion from -668 to -335 and from -335 to -164 resulted in decreases in total promoter activity under all of the light qualities tested. Nevertheless, in At-glw protoplasts the -164 deletion retained high promoter activity under all of the light qualities and still displayed more than 30-fold light induction. Further deletion to -61 abolished activity of the promoter, indicating the presence of critical sequences between -164 and -61. It is this region that contains the putative LRU^{*AtCHS*}. The -164 promoter is therefore defined operationally as the minimal light responsive promoter.

Interestingly, there was no significant difference observed with any of the fusion constructs in the relative effectiveness of UV-B and UV-A/blue light. These results indicate that responsiveness to different light qualities is not mediated by different regions of the promoter.

4.1.2 Deletion Analysis of the *AtCHS* Promoter in At-7 Protoplasts

We decided to try to reproduce these results in a different wild type *A. thaliana* cell culture. This was because we wished to establish whether the use of *A. thaliana*

Figure 4.1 Schematic representation of the *AtCHS* promoter indicating the sizes of promoter deletions

All 5' *AtCHS* promoter deletions are translationally fused to the *uidA* open reading frame encoding GUS. The sequence of the first 24 codons of the open reading frame encoding the fusion protein are shown. The underlined nucleotides are the beginning of the *uidA* coding sequence encoding the GUS reporter gene. The fusions have a common nopaline synthase gene 3' untranslated sequence downstream of the coding sequence (not shown). Positions of the promoter deletions made from position -1972 to -61 of the *AtCHS* promoter relative to the transcription start site are indicated by arrows. The ACE and MRE sequence elements of the LRU are indicated as a vertically striped box and a horizontally striped box respectively. The position of a G-box-like element is represented by a diagonally striped box. The transcriptional start is represented by a right-angled arrow and the *uidA* reporter gene is represented by an unshaded rectangle.

ATG GTG ATG GCT GGT GCT TCT TCT TTG GAT GAG ATC AGA CAG GCT CAG AGA GCT GAT GGA CCT GCA GGC CGG

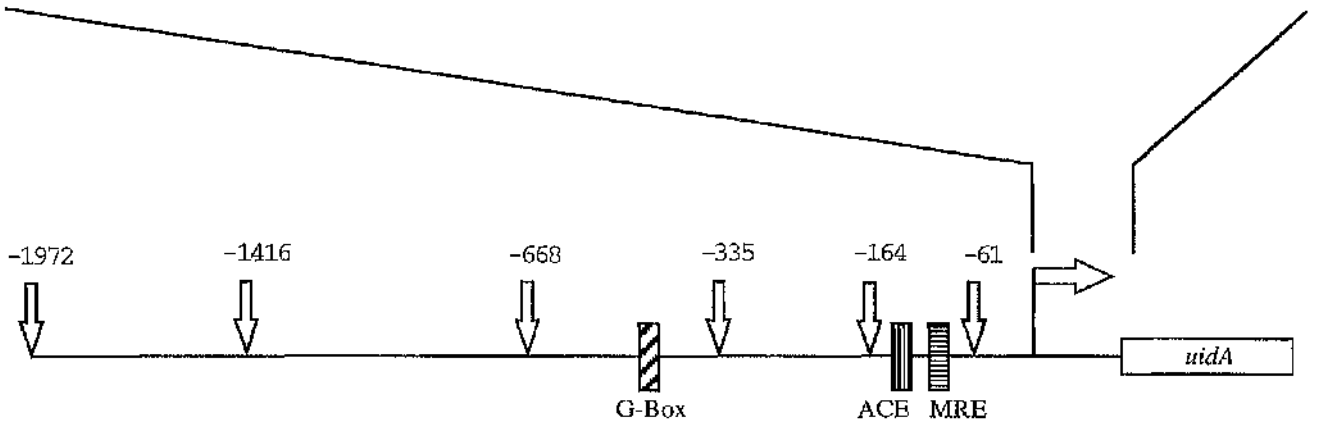
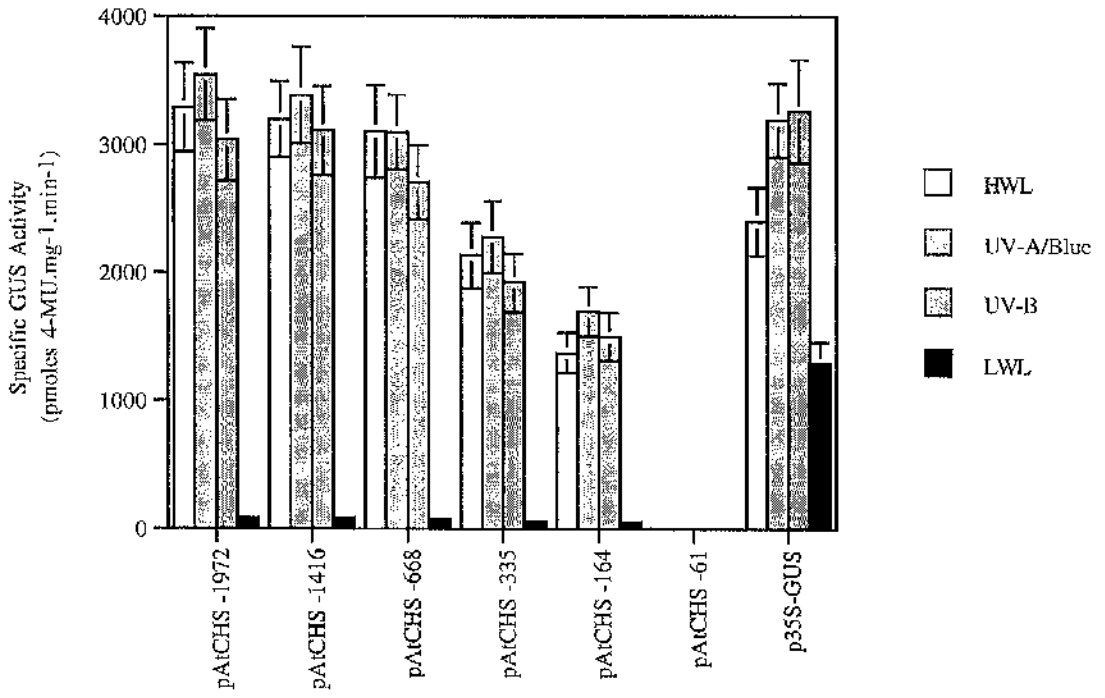


Figure 4.2 Transient expression analysis of *AtCHS* promoter deletion constructs in *At-glw* protoplasts

At-glw protoplasts were transfected with 20 μg of the indicated construct and transferred to HWL ($100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light), UV-A/Blue ($80 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), UV-B ($3 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) or LWL ($20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light) and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



protoplasts derived from different cell cultures would give comparable results. This is important because different cell lines of *A. thaliana* are being used in many different laboratories for the analysis of *A. thaliana* promoters.

The At-7 cell line was white to light-brown in appearance and behaved similarly in several respects to cultured parsley cells of the cell line Pc5 (Korfhage *et al.*, 1994; Trezzini *et al.*, 1993). It had previously been shown that this cell culture also responded to an increased fluence rate of white light or UV-containing white light with similar kinetics of induced *AtCHS* gene activity (Block *et al.*, 1990). Northern analysis had revealed that transcript accumulation showed very similar kinetics both in At-7 protoplasts and At-glw protoplasts following illumination and resembled those reported for intact At-glw cells (Christie and Jenkins, 1996). A method of transient expression analysis had already been developed using protoplasts from At-7 cells in Dr Bernd Weisshaar's laboratory in Cologne. These assays were performed in a very similar manner to those involving the At-glw protoplasts (see Sections 2.19 to 2.23).

The deletion series of constructs was analysed for a second time by transfection into At-7 protoplasts and then either incubated under high fluence rate UV-containing white light or in darkness for 8 h. As shown in Figure 4.3, assays performed using At-7 protoplasts produced very similar expression data to those performed with At-glw protoplasts. All graphs showing specific GUS activity in At-7 protoplasts are presented in a format where columns shown with depth. This is in order to set them apart from graphs representing transient expression studies using At-glw protoplasts which are presented without the columns shown with depth. In At-7 protoplasts the *AtCHS* promoter deletions were, again, inactive in darkness. Deletion of sequences between -1972 and -668 showed no significant decrease in promoter activity under UV-containing white light. We also observed that deletion from -668 to -335 and from -335 to -164 resulted in decreases in total promoter activity under UV-containing white light. As in At-glw protoplasts, in At-7 protoplasts the -164 deletion retained high promoter

activity under inductive conditions and displayed more than 400-fold light induction. Once again deletion to -61 abolished promoter activity suggesting the presence of critical sequences between -164 and -61.

These results suggest that we can indeed use *A. thaliana* protoplasts derived from different cell cultures to perform transient expression assays and produce comparable results. In addition, this experiment using At-7 protoplasts appears to confirm our previous suggestion that the -164 promoter can be defined operationally as the minimal light-responsive promoter.

4.2 FUNCTION OF THE LRU IN UV/BLUE LIGHT RESPONSIVENESS

4.2.1 Production of Constructs for Mutational Analysis of the *AtCHS* Promoter

The above data indicated that sequences important for UV/blue light induction were in the region between -164 and -61 in the *AtCHS* promoter. This is the region of the promoter referred to as the putative LRU^{*AtCHS*} and contains sequences similar to the LRU-1 in the *PcCHS* promoter. In order to examine this further we decided to produce constructs containing block mutations in the putative *cis*-acting elements equivalent to ACE^{*PcCHS*} and MRE^{*PcCHS*} for analysis in the transient expression systems.

The block mutation constructs were derived from the minimal light responsive (-164) promoter construct. The mutations were introduced into the wild type sequence using the "megaprimer" method described by Landt *et al.* (1990) and Sarkar and Sommer (1990). The different block mutations were combined into a single plasmid by taking advantage of the *Bgl*III site located in the *AtCHS* promoter between the putative

Figure 4.3 Transient expression analysis of *AtCHS* promoter deletion constructs in *At-7* protoplasts

At-7 protoplasts were transfected with 20 μ g of the indicated construct and transferred to either darkness or UV-containing white light (four bulbs TL 18W/29 (warm white light; Philips Licht, Hamburg, Germany), three bulbs TL 18W/18 blue (Philips), and two bulbs Sylvania blacklight blue (OSRAM Sylvania, Munich, Germany)) and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for standardised GUS values for six experiments. GUS values were standardised using a luciferase internal standard. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.

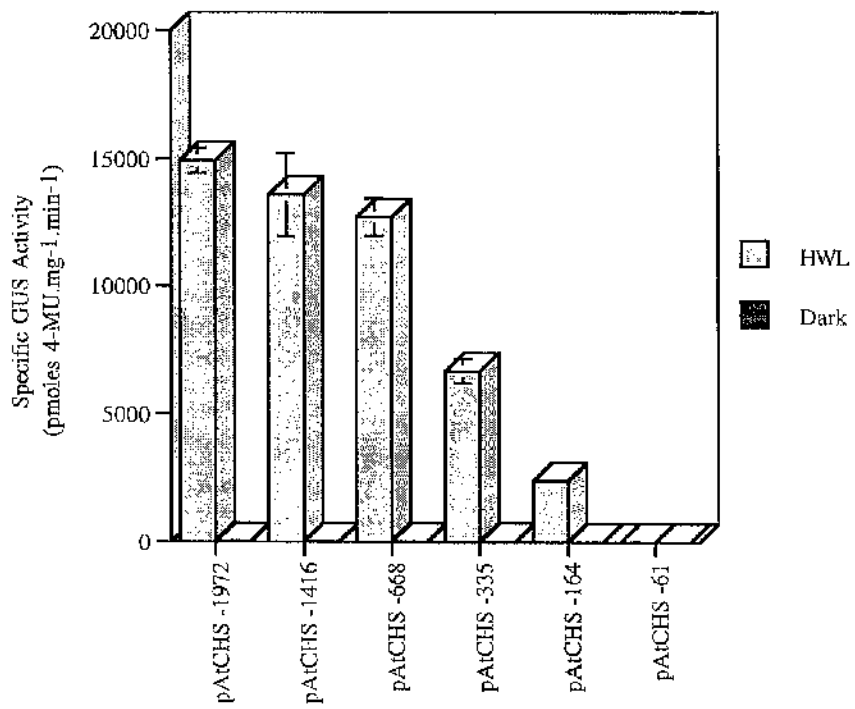


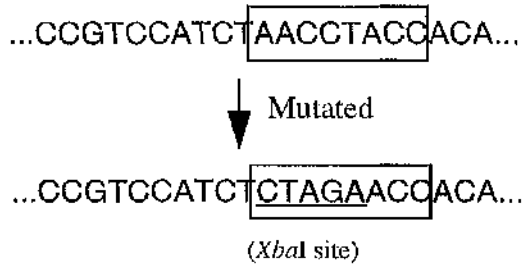
Figure 4.4 Sequences of MREm and ACEm block mutations and schematic representation of mutated -164 bp promoter constructs

A. The nucleotide changes in the MRE and the ACE involved in producing block mutations (MREm and ACEm) are shown. The core sequence of both elements are highlighted by boxes. Both block mutations result in the production of a new restriction enzyme site in the sequence element and these are highlighted by underlining.

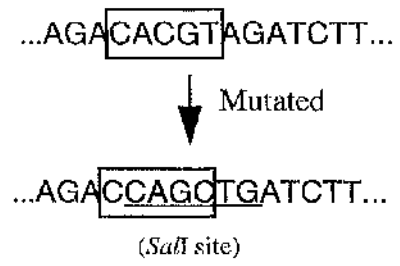
B. All -164 bp *AtCHS* promoters shown are fused to the *uidA* open reading frame encoding GUS. The fusions have a common nopaline synthase gene 3' untranslated sequence downstream of the coding sequence (not shown). The ACE and MRE sequence elements of the LRU are indicated as a shaded box and a striped box respectively and block mutations are represented by a shaded jagged circle.

A

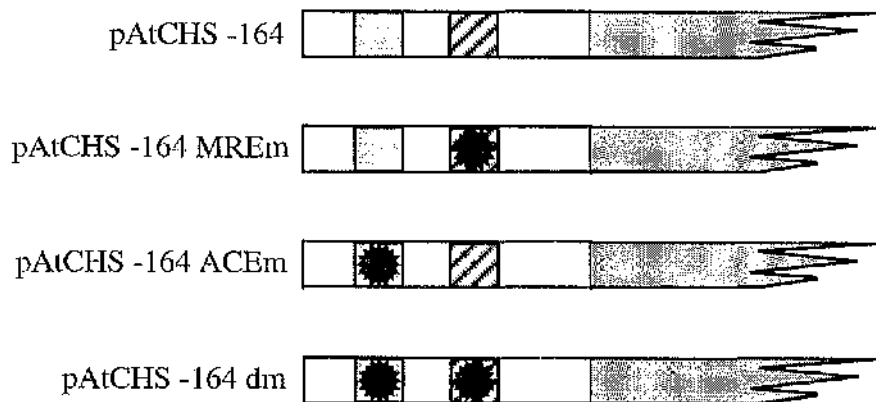
MRE BLOCK MUTATION



ACE BLOCK MUTATION



B



ACE^{AtCHS} and MRE^{AtCHS} sequences. All of these plasmid constructions were verified by sequencing to exclude the introduction of mutations during PCR or at the cloning sites. Details of the cloning procedures used for all of the block mutation constructs are given in Chapter 2. The nucleotide sequence of the block mutations introduced and a diagram of the constructs produced can be seen in Figure 4.4.

The cloning procedures used to prepare the pAtCHS-164 ACE^m construct are outlined in Figure 4.5. A PCR was performed with pAtCHS-164 as a template with primers designed to introduce a block mutation into the ACE sequence and amplify the upstream region of the -164 bp promoter. The PCR products were visualised by gel electrophoresis (Figure 4.5 A) and the 78 bp product was gel purified. Restriction enzymes (*Bgl*II and *Hind*III) whose target sequences were incorporated into the primers were used to digest the 78 bp fragment to produce an insert for ligation into a vector prepared by digesting pAtCHS-164 with the same enzymes. The subsequent ligation resulted in the directional cloning of the insert containing the mutated ACE sequence into the -164 bp promoter region of the prepared vector. The new pAtCHS-164 ACE^m construct was amplified in *E. coli* and the promoter region, including the ligation sites, was sequenced. The pAtCHS-164 MRE^m construct was prepared following a similar strategy by Ulrike Hartmann at the Max Planck Institute in Cologne.

The pAtCHS-164 dm (double mutant) construct was prepared by utilising the *Bgl*II restriction site between the MRE and ACE sequence elements of the promoter. This cloning procedure is outlined in Figure 4.6. Restriction digests of pAtCHS-164 ACE^m and pAtCHS-164 MRE^m with *Bgl*II and *Sca*I to prepare the insert and vector, respectively, for ligation. This ligation resulted in a -164 bp promoter containing block mutations in both the ACE and MRE sequence elements. This plasmid was amplified in *E. coli*, the ligation sites sequenced and designated pAtCHS-164 dm.

Figure 4.5 Schematic representation of the cloning procedures used to prepare pAtCHS -164 ACE_m

A. Photograph of a 1.5 % agarose gel stained with EtBr and visualised in UV light. Lane 1 is 1 kb DNA ladder. The sizes of prominent bands are labelled. Lane 2 is 5 µl of a PCR reaction used to amplify a region of the *AtCHS* promoter and introduce a block mutation into the ACE sequence element. The 78 bp PCR product is indicated.

B. Schematic representation of the cloning procedures used to prepare pAtCHS -164 ACE_m. Plasmids are represented by a circle, the transcriptional start site is indicated by a right-angled arrow, MRE and ACE sequence elements are represented by rectangles, PCR primers are indicated by single-headed arrows, the mutated ACE sequence is represented by a shaded star and relevant restriction sites are labelled. The PCR primers indicate the region of the plasmid amplified by “megaprimer” PCR and the resulting 78 bp PCR product contains an ACE_m and is restriction digested with *Hind*III and *Bgl*II. This fragment is ligated into a vector prepared by restriction digesting pAtCHS -164 with *Hind*III and *Bgl*II to produce the pAtCHS -164 ACE_m plasmid.

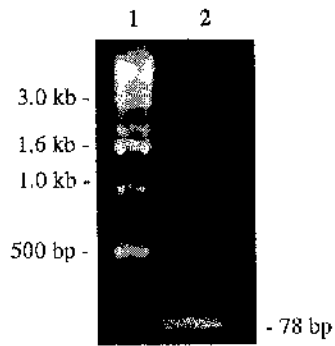
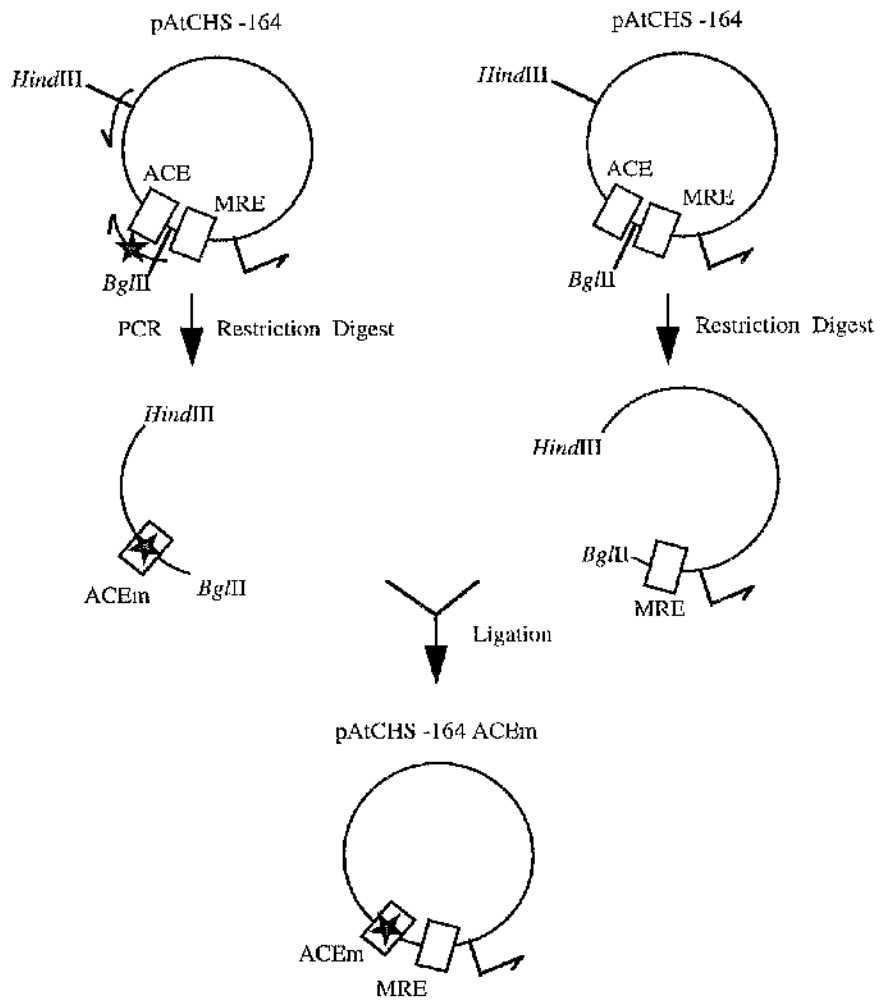
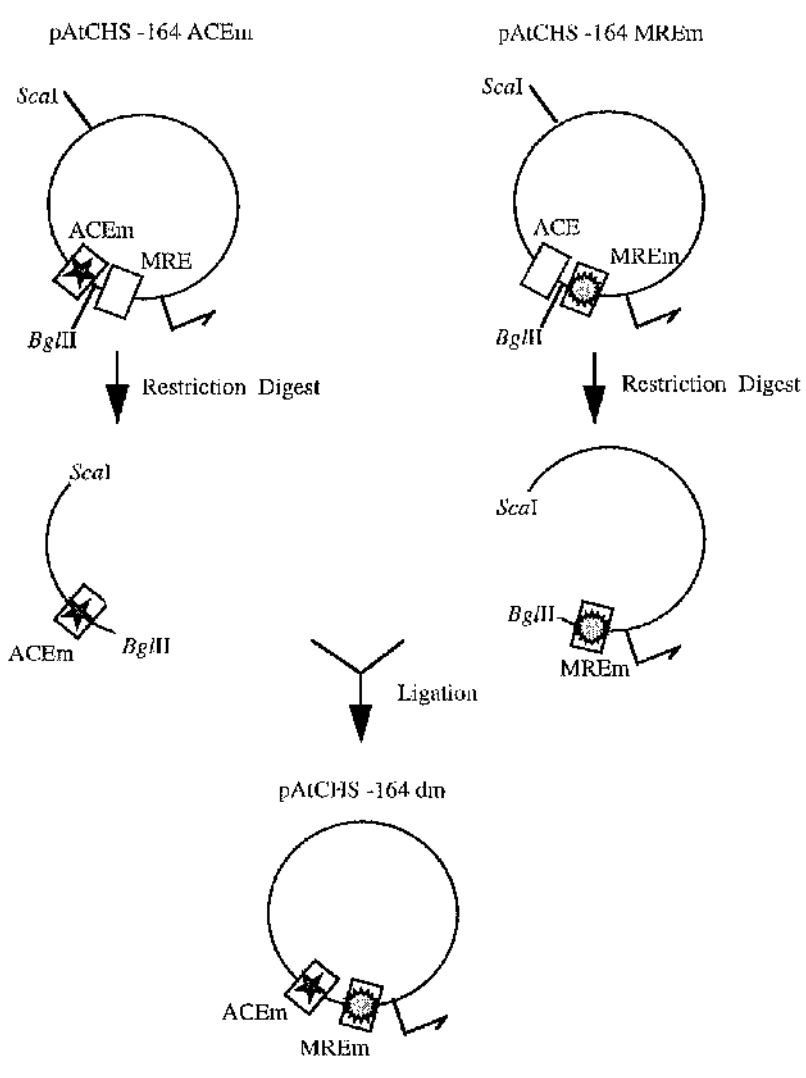
A**B**

Figure 4.6 Schematic representation of the cloning procedures used to prepare pAtCHS -164 dm

Schematic representation of the cloning procedures used to prepare pAtCHS -164 dm. Plasmids are represented by a circle, the transcriptional start site is indicated by a right-angled arrow, MRE and ACE sequence elements are represented by rectangles, the mutated ACE sequence is represented by a shaded star, the mutated MRE is represented by a shaded jagged circle and relevant restriction sites are labelled. Restriction digest of pAtCHS -164 ACEm with *ScaI* and *BglII* produced an insert containing ACEm and the upstream -164 bp *AtCHS* promoter region. Restriction digest of pAtCHS -164 MREm with *ScaI* and *BglII* produced a vector containing MREm, the downstream *AtCHS* promoter region fused to the reporter gene. Ligation of insert and vector produced the pAtCHS -164 dm plasmid.



4.2.2 Mutational Analysis of the Minimal *AtCHS* Promoter in *At-7* Protoplasts

In order to investigate the effects of these mutations in the context of the minimal promoter we performed transient expression assays using the block mutation constructs in protoplasts derived from *At-7* cells. The results of this analysis are shown in Figure 4.7. Mutation of either of the *cis*-acting elements greatly reduced the level of promoter

activity in protoplasts incubated under UV-containing white light, although a low level of light induction was still detectable. Further, mutation of both elements resulted in total inactivation of the -164 promoter. These observations indicate that the putative ACE^{AtCHS} and MRE^{AtCHS} sequences are necessary for *AtCHS* light regulation in the context of the minimal promoter.

4.2.3 Mutational Analysis of the Minimal *AtCHS* Promoter in *At-glw* Protoplasts

In order to confirm the above observations and investigate how the induction by UV-B and UV-A/blue is affected by block mutation of the putative ACE^{AtCHS} and MRE^{AtCHS} elements, we performed transient expression analysis using the same constructs in protoplasts prepared from the *At-glw* cell culture. As can be seen in Figure 4.8, the results were very similar to those obtained using the *At-7* transient expression system. Once more we can see that mutation of either of the *cis*-acting elements greatly reduced the level of promoter activity in protoplasts incubated under high intensity white light, UV-A/blue light and UV-B light, although light induction was still detectable at greatly reduced levels. No difference in the relative effectiveness of UV-B and UV-A/blue light was observed with either block mutation, indicating that neither element mediates a response preferentially to a particular light quality. Again we observed that mutation of both elements abolishes activity of the -164 promoter under all of the light conditions tested. These results show that the ACE^{PcCHS} and MRE^{PcCHS} -like sequences are

Figure 4.7 Transient expression analysis of -164 bp *AtCHS* promoter constructs in At-7 protoplasts

At-7 protoplasts were transfected with 20 μg of the indicated construct and transferred to either darkness or UV-containing white light (four bulbs TL 18W/29 (warm white light; Philips Licht, Hamburg, Germany), three bulbs TL 18W/18 blue (Philips), and two bulbs Sylvania blacklight blue (OSRAM Sylvania, Munich, Germany)) and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for standardised GUS values for six experiments. GUS values were standardised using a luciferase internal standard. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.

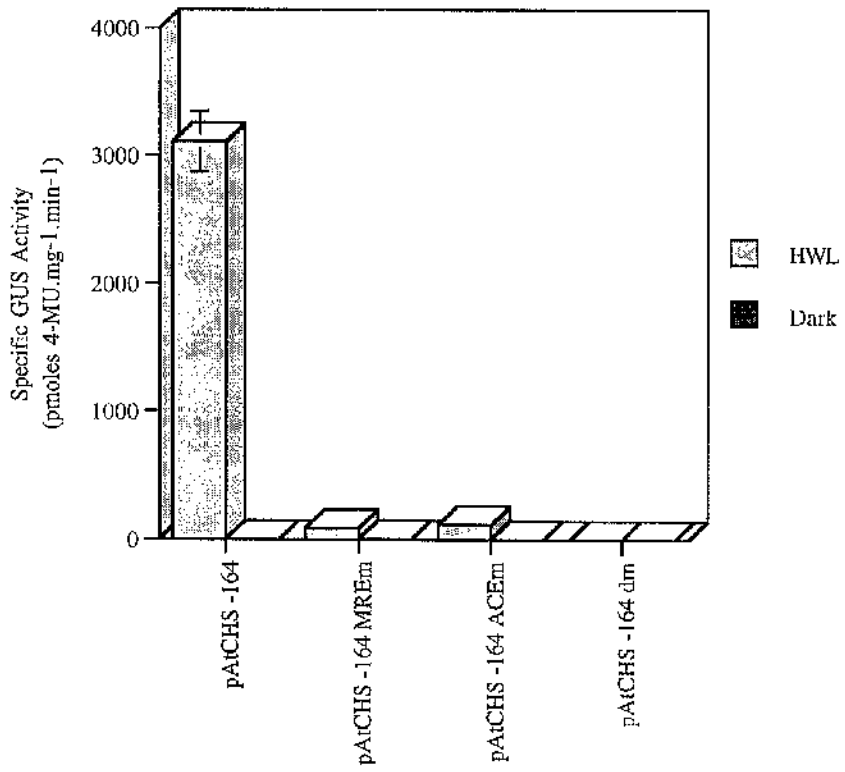
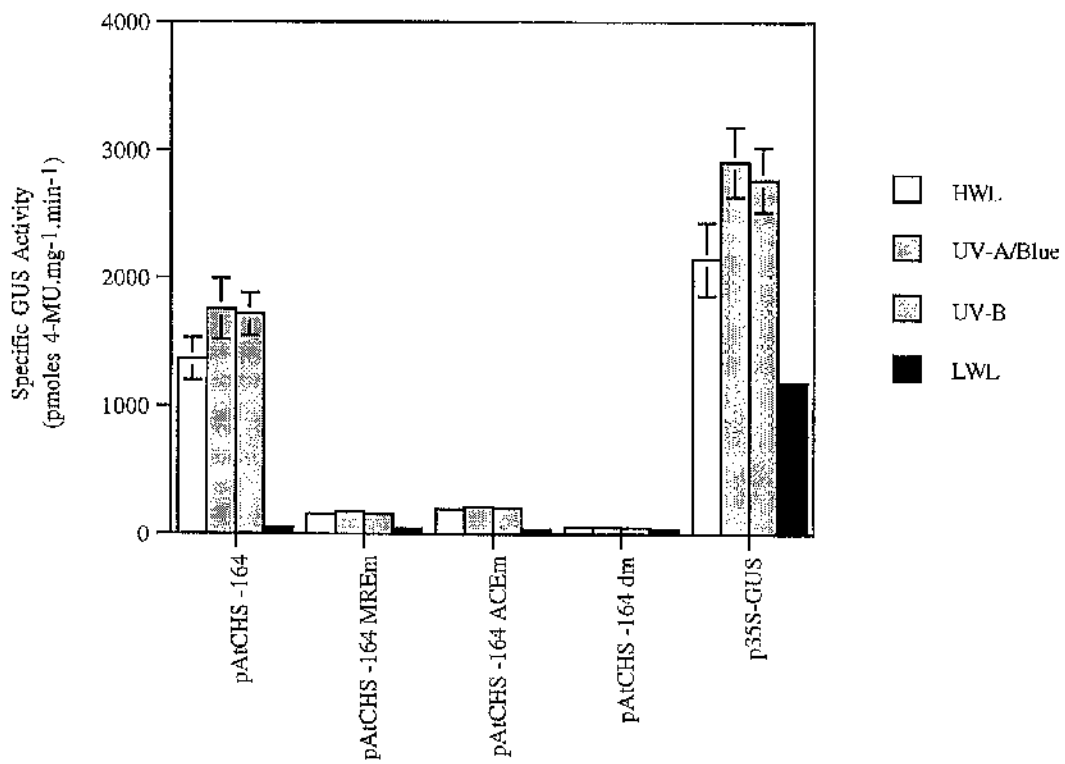


Figure 4.8 Transient expression analysis of -164 bp *AtCHS* promoter constructs in *At-glw* protoplasts

At-glw protoplasts were transfected with 20 μg of the construct indicated and transferred to HWL ($100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light), UV-A/Blue ($80 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), UV-B ($3 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) or LWL ($20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light) and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



necessary for both UV-B and UV-A/blue light induction of the *AtCHS* promoter. We will therefore refer to these newly defined *cis*-acting elements as ACE^{*AtCHS*} and MRE^{*AtCHS*}.

4.3 THE ROLE OF THE LRU^{*AtCHS*} IN LIGHT RESPONSIVE PROMOTER ACTIVITY

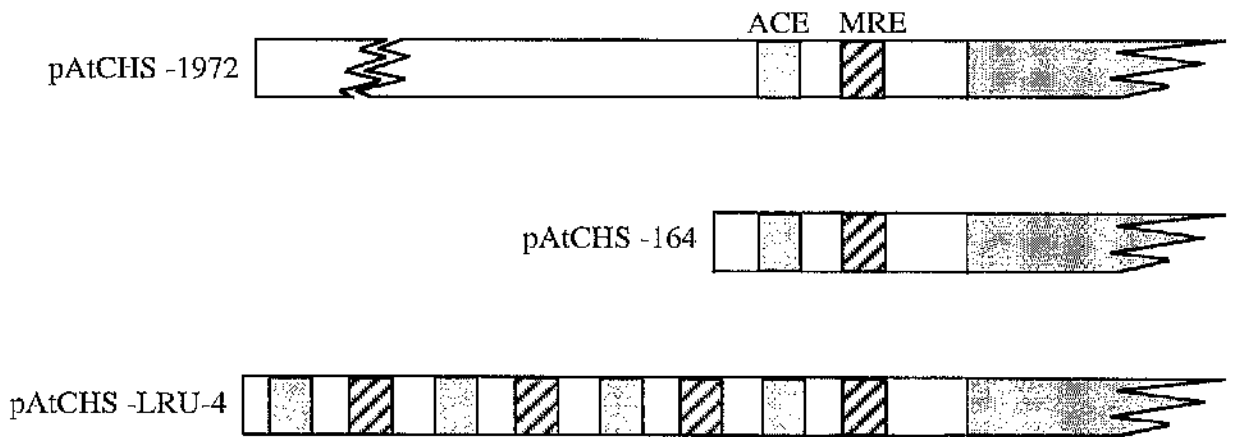
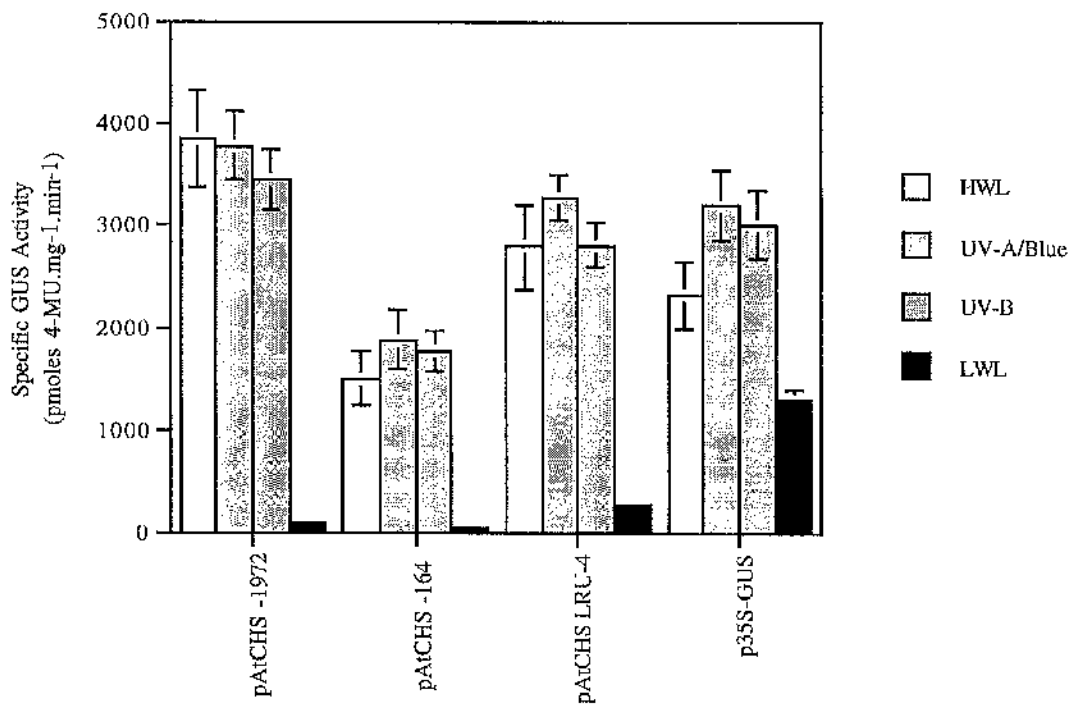
We decided to further test the role of the ACE^{*AtCHS*} and MRE^{*AtCHS*} sequence elements in light dependent promoter activation by performing a gain of function experiment. To do this we performed transient expression assays with *At*-glw protoplasts using a construct provided by Ulrike Hartmann at the Max Planck Institute for Plant Breeding in Cologne. This plasmid contained a promoter which had positions -59 to -106 of the *AtCHS* promoter fused as a tetramer to a -46 35S core promoter containing only the TATA box and initiation site driving expression of the *uidA* coding sequence (Figure 4.9 A). The results of these assays are shown in Figure 4.9 B.

We observed that this *AtCHS* promoter region was able to confer high levels of light-induced expression on the heterologous core promoter in the *At*-glw transient expression system. Expression from the tetramer construct was shown to be almost double that of the minimal -164 promoter but not quite as high as that seen with the full-length -1972 *AtCHS* promoter. In addition, the LRU^{*AtCHS*} tetramer promoter mediated an equivalent response to UV-B and UV-A/blue wavelengths. The only difference in comparison to the authentic promoter was that the levels of dark expression increased slightly. Together with the results from the block mutation constructs (Figures 4.7 and 4.8), these data demonstrate that the LRU^{*AtCHS*} is, in the context of the minimal promoter, both necessary and sufficient to confer UV-B and UV-A/blue light responsive promoter activity.

Figure 4.9 Light regulated LRU tetramer promoter activity in At-glw protoplasts

A. The *AtCHS* promoters shown are fused to the *uidA* open reading frame encoding GUS. The fusions have a common nopaline synthase gene 3' untranslated sequence downstream of the coding sequence (not shown). The ACE and MRE sequence elements of the LRU are indicated as a shaded box and a striped box respectively. The LRU tetramer construct (pAtCHS LRU-4) contains four copies of the LRU from the *AtCHS* promoter in the original orientation fused to the -46 deletion of the *CaMV 35S* promoter.

B. At-glw protoplasts were transfected with 20 μg of the indicated construct and transferred to HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light), UV-A/Blue ($80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$), UV-B ($3 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) or LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.

A**B**

4.4 FUNCTION OF SEQUENCES UPSTREAM OF LRU^{AtCHS} IN LIGHT INDUCTION AND QUANTITATIVE EXPRESSION

The stepwise reduction in light-induced promoter activity in the deletion series (Figures 4.2 and 4.3) suggested that (a) further element(s) in the promoter, upstream of -164 were important in conferring maximal activity. We therefore attempted to define the element(s) involved by, firstly, identifying the region(s) of the promoter in which the additional element(s) are located and secondly by performing mutational analysis of the putative additional element(s).

4.4.1 Production of Constructs to Locate an Additional Light-Responsive Region in the *AtCHS* Promoter

In order to establish which region(s) of the promoter may contain (an) additional sequence element(s) we created a series of 5' promoter deletion in which both *cis*-acting elements in the LRU^{AtCHS} were block mutated, thereby abolishing the function of this light-responsive unit completely. The block mutation constructs were derived from each of the 5' promoter deletion constructs and existing block mutation constructs (4.1.1 and 4.2.1). The cloning procedures used to prepare the double mutant constructs are outlined in Figures 4.10, 4.11 and 4.12.

Figure 4.10 A shows how the pAtCHS-1972 dm plasmid was prepared. The *Bgl*III restriction site between the two sequence elements of the LRU^{AtCHS} was utilised in this strategy. Restriction digests with *Hind*III and *Bgl*III were used to prepare the insert and vector from pAtCHS-1972 ACEm and pAtCHS-1972 MREm (kindly supplied by Ulrike Hartmann) respectively. The resulting insert contained the mutated ACE sequence and the remaining upstream region of the *AtCHS* promoter and the vector

Figure 4.10 Schematic representation of the cloning procedures used to prepare pAtCHS -1972 dm and pAtCHS -1416 dm

A. Schematic representation of the cloning procedures used to prepare pAtCHS -1972 dm. Plasmids are represented by a circle, the transcriptional start site is indicated by a right-angled arrow, MRE and ACE sequence elements are represented by rectangles, the mutated ACE sequence is represented by a shaded star, the mutated MRE is represented by a shaded jagged circle and relevant restriction sites are labelled. Restriction digest of pAtCHS -1972 ACEm with *Hind*III and *Bgl*II produced an insert containing ACEm and the upstream -1972 bp *AtCHS* promoter region. Restriction digest of pAtCHS -1972 MREm with *Hind*III and *Bgl*II produced a vector containing MREm, the downstream *AtCHS* promoter region fused to the reporter gene. Ligation of insert and vector produced the pAtCHS -1972 dm plasmid as shown.

B. The symbols described above are used to represent the cloning procedures used to prepare the pAtCHS -1416 dm plasmid. pAtCHS -1972 dm was restriction digested with *Hind*III and *Xho*I to cut out the downstream 556 bp of the *AtCHS* promoter and the sticky ends blunted using Klenow enzyme. The blunt ends were ligated to produce the pAtCHS -1416 dm plasmid.

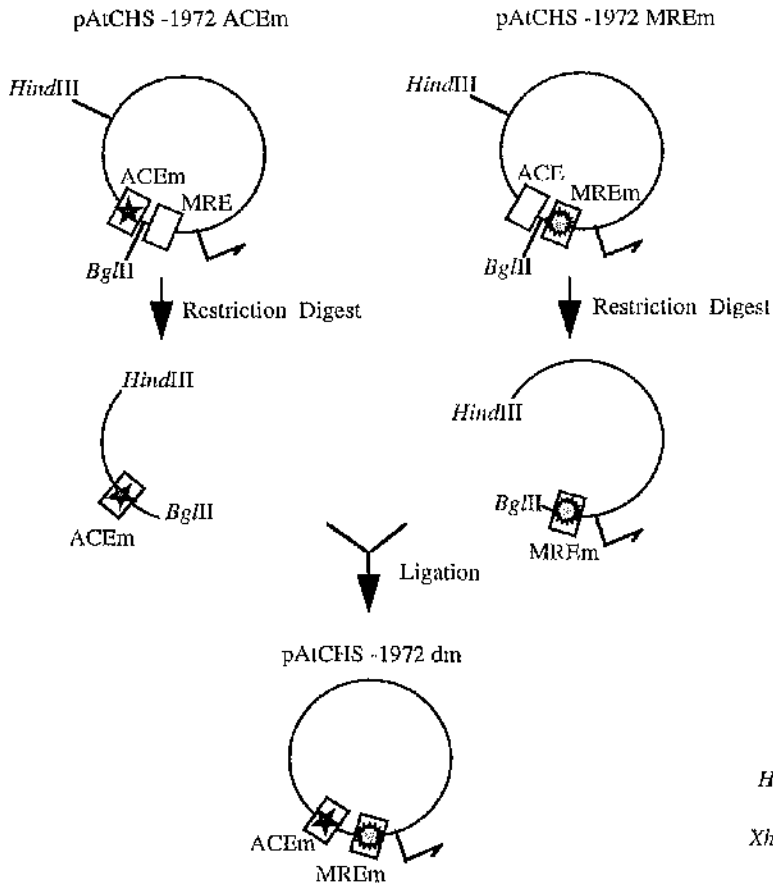
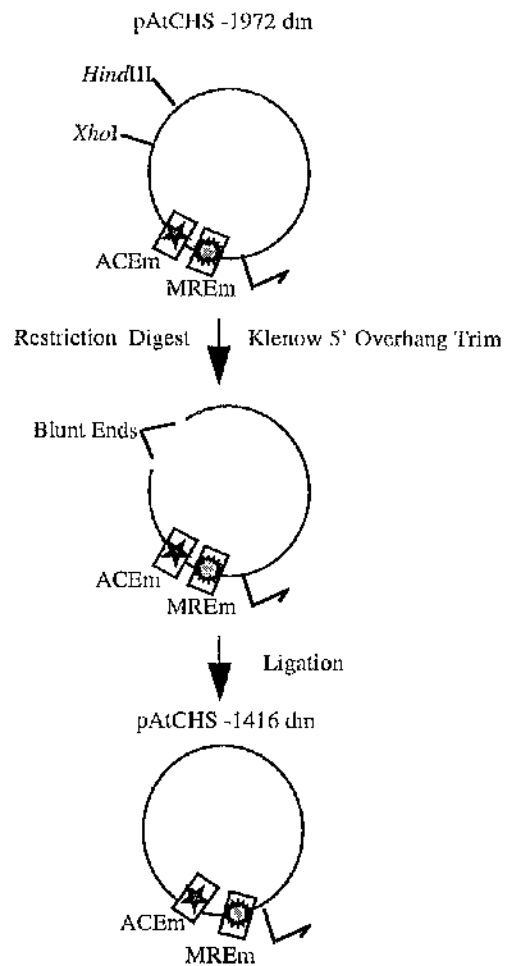
A**B**

Figure 4.11 Schematic representation of the cloning procedures used to prepare pAtCHS -668 dm

A. Photograph of a 1.5 % agarose gel stained with EtBr and visualised in UV light. Lane 1 is 1 kb DNA ladder. The sizes of prominent bands are labelled. Lane 2 is 5 µl of a PCR reaction used to amplify a region of the *AtCHS* promoter and introduce a block mutation into the ACE sequence element. The 604 bp PCR product is indicated.

B. Schematic representation of the cloning procedures used to prepare pAtCHS -668 dm. Plasmids are represented by a circle, the transcriptional start site is indicated by a right-angled arrow, MRE and ACE sequence elements are represented by rectangles, PCR primers are indicated by single-headed arrows, the mutated ACE sequence is represented by a shaded star, the MREm is represented by a shaded jagged circle and relevant restriction sites are labelled. The PCR primers indicate the region of the pAtCHS -668 template amplified by "megaprimer" PCR and the resulting 604 bp PCR product contains an ACEm and the downstream region of the 668 bp *AtCHS* promoter and was restriction digested with *HindIII* and *BglII*. This fragment was ligated into a vector prepared by restriction digesting pAtCHS -1972 MREm with *HindIII* and *BglII*, thus removing the -1972 bp *AtCHS* promoter downstream of the *BglII* site and replacing it with the -668 bp promoter containing ACEm, resulting in the production of the pAtCHS -668 dm plasmid.

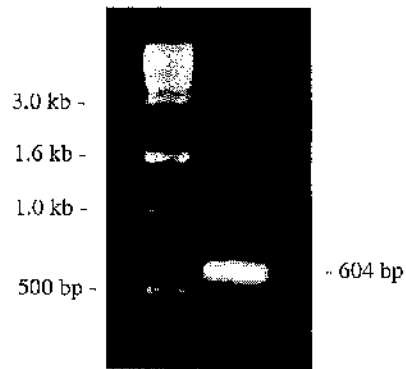
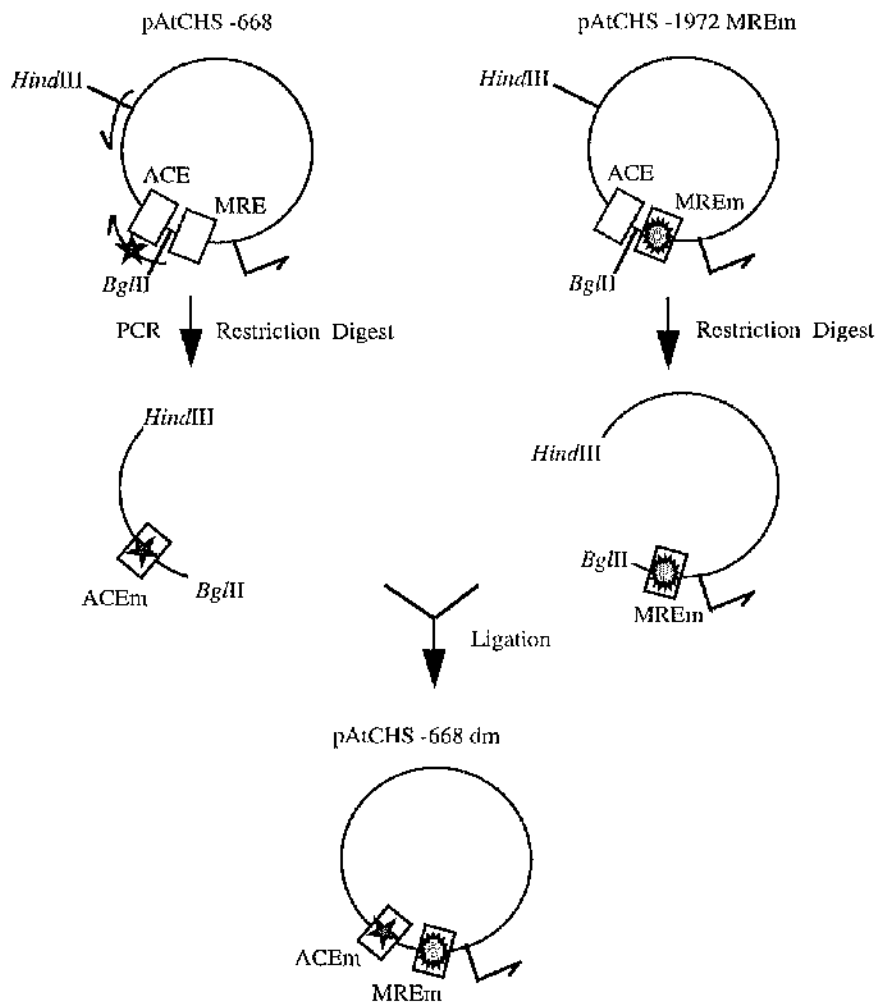
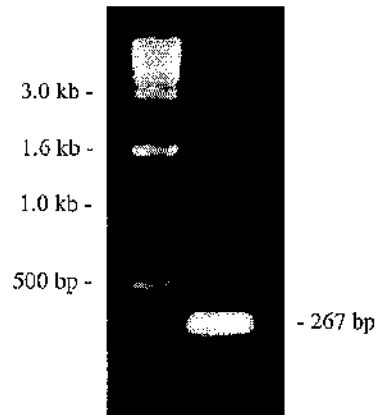
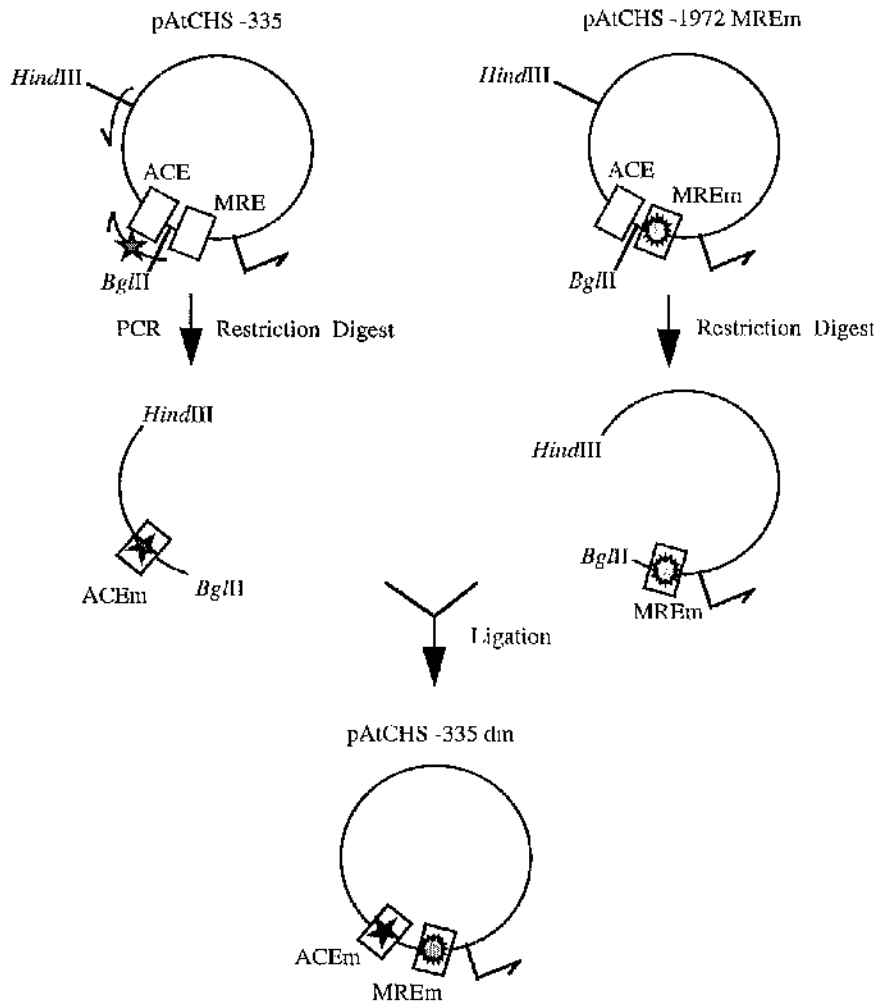
A**B**

Figure 4.12 Schematic representation of the cloning procedures used to prepare pAtCHS -335 dm

A. Photograph of a 1.5 % agarose gel stained with EtBr and visualised in UV light. Lane 1 is 1 kb DNA ladder. The sizes of prominent bands are labelled. Lane 2 is 5 µl of a PCR reaction used to amplify a region of the *AtCHS* promoter and introduce a block mutation into the ACE sequence element. The 267 bp PCR product is indicated.

B. Schematic representation of the cloning procedures used to prepare pAtCHS -335 dm. Plasmids are represented by a circle, the transcriptional start site is indicated by a right-angled arrow, MRE and ACE sequence elements are represented by rectangles, PCR primers are indicated by single-headed arrows, the mutated ACE sequence is represented by a shaded star, the MREm is represented by a shaded jagged circle and relevant restriction sites are labelled. The PCR primers indicate the region of the pAtCHS -335 template amplified by "megaprimer" PCR and the resulting 267 bp PCR product contains an ACEm and the downstream region of the 335 bp *AtCHS* promoter and was restriction digested with *HindIII* and *BglII*. This fragment was ligated into a vector prepared by restriction digesting pAtCHS -1972 MREm with *HindIII* and *BglII*, thus removing the -1972 bp *AtCHS* promoter downstream of the *BglII* site and replacing it with the -335 bp promoter containing ACEm, resulting in the production of the pAtCHS -335 dm plasmid.

A**B**

contained the mutated MRE sequence, the remaining downstream region of the *AtCHS* promoter, transcriptional start and the *CHS-GUS* fusion coding sequence. Ligation of this insert and vector produced a full-length *AtCHS* promoter construct containing both the mutated ACE and the mutated MRE sequences. This construct was sequenced at the ligation sites and designated p*AtCHS*-1972 dm.

The p*AtCHS*-1416 dm construct was derived from the p*AtCHS*-1972 dm plasmid as outlined in Figure 4.10 B. p*AtCHS*-1972 dm was restriction digested with *Hind*III and *Xho*I and the resulting 6.353 kb fragment was gel purified. The 5' overhangs produced by the restriction enzymes were filled in using the Klenow enzyme. The resulting blunt ends were ligated to produce the p*AtCHS*-1416 dm plasmid and sequenced at the ligation site.

The cloning procedures used to make the p*AtCHS*-668 dm plasmid are shown in Figure 4.11 B. A PCR was performed using the p*AtCHS*-668 plasmid as template and using the "megaprimer" method to introduce a ACE_m sequence into the PCR product (Landt *et al.*, 1990; Sarkar and Sommer, 1990). This 604 bp PCR product (see Figure 4.11 A) was gel purified, digested with *Hind*III and *Bgl*II and ligated into a vector prepared by digesting the p*AtCHS*-1972 MRE_m plasmid with the same restriction enzymes and gel purification. The resulting construct had a -668 bp *AtCHS*-promoter containing block mutations in both the ACE and MRE sequence elements. The ligation sites and the promoter region produced by PCR were sequenced and the construct designated p*AtCHS*-668 dm.

The p*AtCHS*-335 dm plasmid was produced in a manner similar to that described above for the p*AtCHS*-668 dm construct. However, the p*AtCHS*-335 plasmid was used as a template for the "megaprimer" PCR to produce the insert, which contains an ACE_m, for ligation into a vector containing MRE_m prepared from the p*AtCHS*-1972 MRE_m plasmid. This cloning procedure and the 267 bp PCR product are shown in Figure 4.12

A/B. The ligation sites and the promoter region produced by PCR were sequenced and the construct designated p*AtCHS*-335 dm.

4.4.2 Location of an Additional Light-Responsive Region in the *AtCHS* Promoter

This series of double mutant constructs are shown with the corresponding 5' deletion construct in figure 4.13. These constructs were analysed in transient expression assays in protoplasts derived from *At-glw* cells. The results of these assays are shown in Figure 4.14. This data shows that the -668 double mutant promoter quite clearly remains light-responsive. In contrast, the -335 and -164 double mutant promoters essentially showed a complete loss of light responsiveness. This indicates that there is an additional light-responsive region located between positions -335 and -668 of the *AtCHS* promoter. In this experiment, as in previous analyses, we were again unable to detect any difference in responsiveness to UV-B and UV-A/blue light qualities with any of the constructs tested. These results indicate that sequences upstream of the LRU^{*AtCHS*} are able to confer light responsiveness and that they mediate the same qualitative response to different light qualities as the LRU^{*AtCHS*}. However, this analysis does appear to indicate that there is a stepwise reduction in light-induced promoter activity between the -1972, -1416 and the -668 double mutant promoters. This stepwise reduction in promoter activity observed with the longer double mutant constructs appears to indicate that there are several regions of the promoter which exert quantitative effects on promoter activity.

4.4.3 Mutational Analysis of a Putative Light-Responsive Region Upstream of the LRU^{*AtCHS*}

One of the three potential *cis*-acting elements in the *AtCHS* promoter previously

Figure 4.13 Schematic representation of the *AtCHS* promoter deletion constructs and double mutant constructs

All 5' *AtCHS* promoter deletions are fused to the *uidA* open reading frame encoding GUS. The fusions have a common nopaline synthase gene 3' untranslated sequence downstream of the coding sequence (not shown). Promoter deletions from position -1972 to -61 of the *AtCHS* promoter relative to the transcription start site together with the corresponding double mutant promoter are shown. The ACE and MRE sequence elements of the LRU are indicated by a shaded box and a striped box respectively. Block mutations are represented by a shaded jagged circle

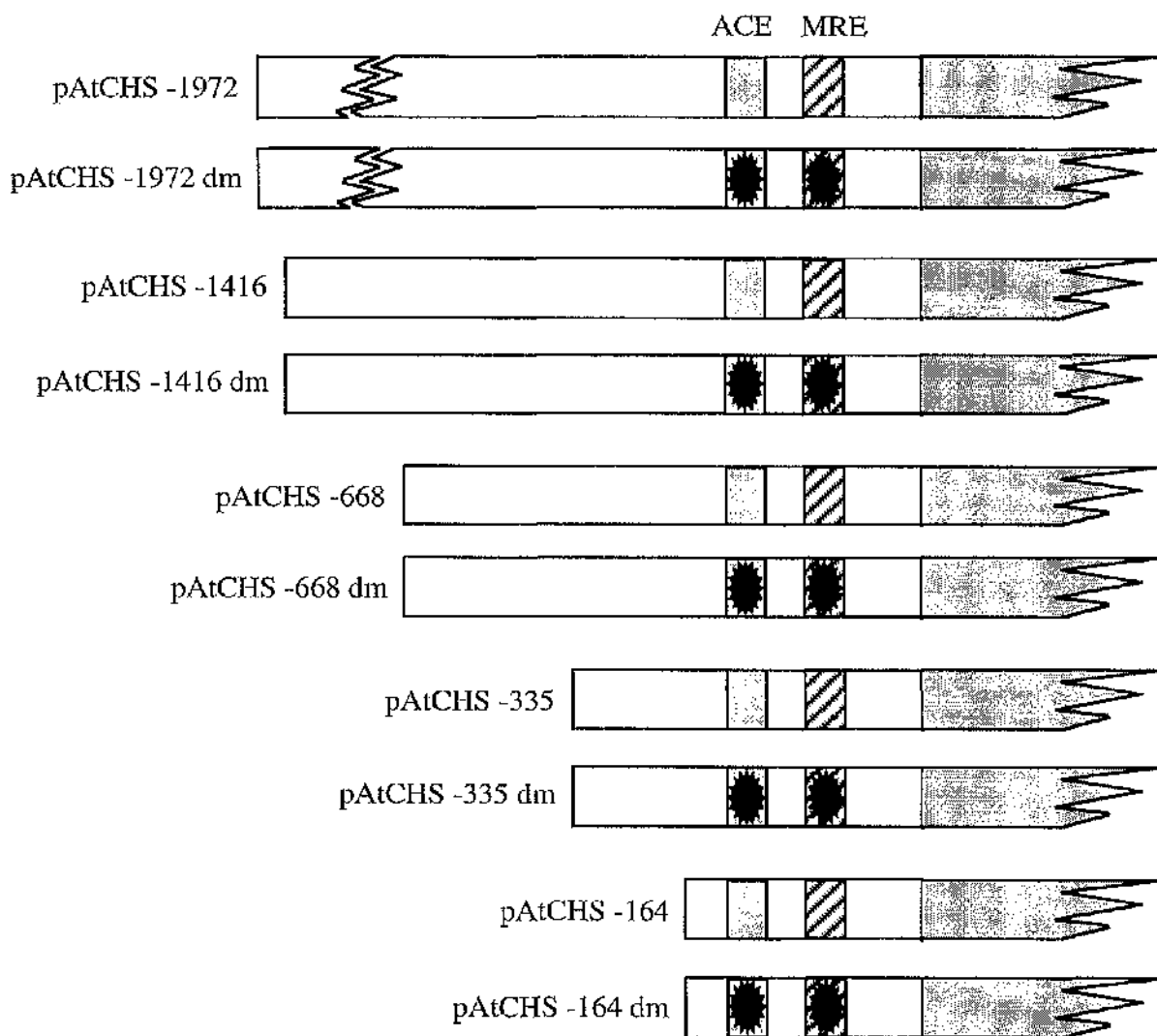
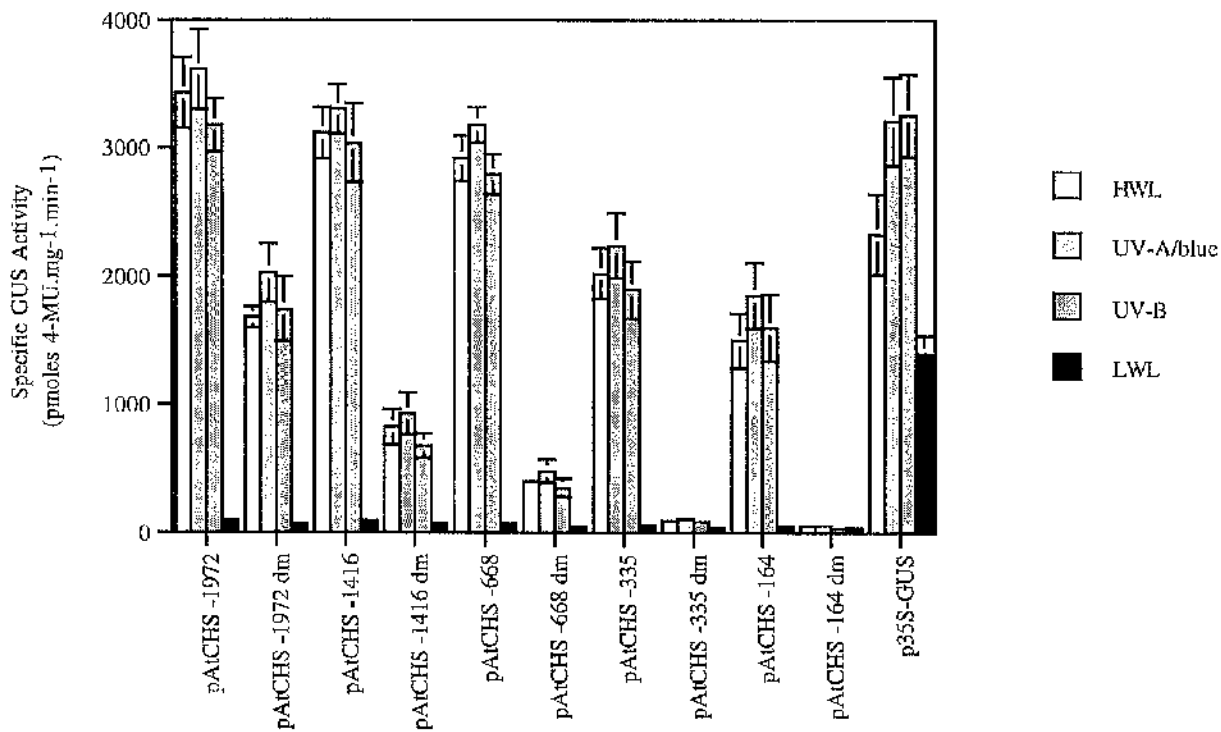


Figure 4.14 Transient expression analysis of the double mutant constructs in *At-glw* protoplasts

At-glw protoplasts were transfected with 20 μg of the indicated construct and transferred to HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light), UV-A/Blue ($80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$), UV-B ($3 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) or LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



identified by sequence comparison was a G-box-like potential ACE around position -442. Since the transient expression analysis with the double mutant constructs indicated the presence of a light-responsive region located between positions -335 and -668 of the *AtCHS* promoter in addition to the LRU^{*AtCHS*}, we decided to investigate whether this G-box-like sequence might be involved in the light responsiveness of the promoter. In order to address this question, we used constructs with the G-box-like sequence mutated in the context of both the -668 promoter and the -668 promoter with the double mutation in the LRU^{*AtCHS*}. These constructs were produced by Ulrike Hartmann at the Max Planck Institute for Plant Breeding in Cologne and are shown in diagrammatic form in Figure 4.15.

Transient expression analysis revealed that mutation of the G-box-like element in the non-mutant -668 promoter had no detectable effect on expression (Figure 4.16). It also appears that there was very little difference in promoter activity driven by the double mutant promoter (-668dm) whether or not the G-box-like sequence was intact. Furthermore, the triple mutant promoter (-668tm) displayed the same light responsiveness to both UV-B and UV-A/blue light qualities as did the double mutant promoter. We therefore conclude that the lone G-box-like sequence does not appear to be functional in the light regulation of the promoter. Other element(s) in the -335 to -668 region of the promoter must confer the residual degree of light-regulated expression detected in the -668 double and triple mutant promoter constructs.

4.5 DISCUSSION

The development and application of an efficient homologous transient expression system has allowed us to functionally dissect the promoter elements of the *Arabidopsis CHS* gene. The light responsive nature of protoplasts produced from both the *At-glw*

**Figure 4.15 Schematic representation of constructs for analysis of
G-box-like element in the -668 bp promoter**

All -668 bp *AtCHS* promoters are fused to the *uidA* open reading frame encoding GUS. The fusions have a common nopaline synthase gene 3' untranslated sequence downstream of the coding sequence (not shown). Promoter deletions from position -1972 to -61 of the *AtCHS* promoter relative to the transcription start site together with the corresponding double mutant promoter are shown. The ACE and MRE sequence elements of the LRU are indicated by a lightly shaded box and a striped box respectively. The G-box-like element is represented by a darkly shaded box. Block mutations are represented by a shaded jagged circle

G-box-like Element ACE MRE

pAtCHS -668



pAtCHS -668 gm



pAtCHS -668 dm

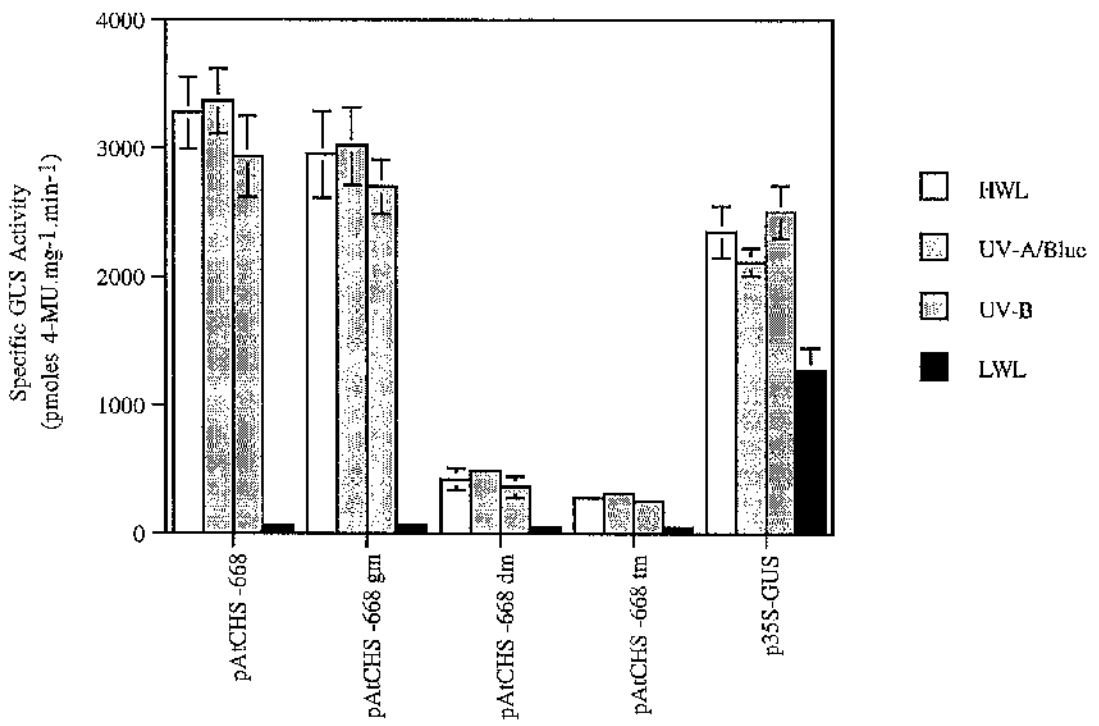


pAtCHS -668 tm



**Figure 4.16 Transient expression analysis of G-box-like element in the
-668 bp *AtCHS* promoter**

At-glw protoplasts were transfected with 20 μg of the construct indicated and transferred to HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light), UV-A/Blue ($80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$), UV-B ($3 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) or LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



and At-7 cell culture has facilitated detailed analysis of the light responsive nature of the defined promoter elements. This analysis has allowed us to make the following observations about the *AtCHS* promoter:

1. The minimal -164 bp *At CHS* promoter is sufficient for light regulation.
2. The ACE^{*AtCHS*} and MRE^{*AtCHS*} are necessary for light regulation within the minimal promoter.
3. The LRU^{*AtCHS*} is, in the context of the minimal promoter, both necessary for light-dependent induction and sufficient to confer light-responsive promoter activity.
4. Sequences upstream of the LRU^{*AtCHS*} are able to confer light responsiveness and mediate the same qualitative response to different light qualities as the LRU^{*AtCHS*}.
5. The G-box element (around position -442) does not appear to be functional in the light regulation of the promoter.
6. No preferential responsiveness to UV-B or UV-A/blue light was observed with an promoter element.

CHAPTER 5: THE ROLE OF CRYPTOCHROMES IN THE CONTROL OF *AtCHS* REGULATION

5.1 INTRODUCTION

In recent years, as outlined in Chapter 1, two photoreceptors known as cryptochromes, CRY1 and CRY2 have been cloned using molecular techniques (Ahmad and Cashmore, 1993; Ahmad *et al.*, 1998a). The *CRY2* gene was cloned more recently than *CRY1* and as such is less well characterised. In contrast, the CRY1 photoreceptor has been functionally well defined and is clearly involved in a number of extension-growth responses, for example inhibition of hypocotyl elongation, and in the control of expression of a number of genes, for example *rbcS* (Jackson and Jenkins, 1995; Conley and Shih, 1995).

Several questions, however, remain to be addressed with regard to the role of cryptochromes in the control of *AtCHS* expression. It is clear that in mature leaves, expression is controlled mainly by UV-A/blue and UV-B light (Jackson and Jenkins, 1995; Ahmad *et al.*, 1995). Phytochrome regulation of *AtCHS* gene expression appears to be confined to seedlings less than 6 days old (Kaiser *et al.*, 1995). Experiments with *hy4* mutants showed reduced *AtCHS* expression in blue light, loss of expression in UV-A light and unaltered expression in UV-B light (Fuglevand *et al.*, 1996). These results suggest that CRY1 mediates part of blue, all of UV-A and none of UV-B light induced expression of *AtCHS*. Therefore, at least one other photoreceptor is involved in mediating blue and UV-B light induced expression. The problem with experiments using mutants is functional redundancy, as seen in several phytochrome mutant studies. Functional overlap between photoreceptors can mask the effects of a particular mutation. Therefore, in experiments with *hy4* mutants, it is possible that CRY2 is functioning to mediate residual blue light induced *AtCHS* expression and UV-

B light induced expression. In addition, Fuglevand *et al.* (1996) reported a synergistic induction of *AtCHS* expression in response to either UV-A and UV-B light or to blue and UV-B light. These synergistic responses were shown to be distinct and additive and it was shown that neither synergism was affected in *hy4* plants. However, again, functional redundancy between photoreceptors may explain these observations. The availability of *cry2 (fha1)* mutants allows us to address the following questions:

1. Does CRY2 play a role in the residual blue light-induced *AtCHS* expression?
2. Does CRY2 play a role in mediating UV-B light-induced *AtCHS* expression?
3. Is CRY2 involved in mediating the UV and blue synergistic induction of *AtCHS* expression?

Several different approaches were employed to investigate the role of cryptochromes, CRY1 and CRY2, in the regulation of *AtCHS* promoter activity. Gain of function transient expression analysis suggests that CRY1 is likely to be the primary photoreceptor mediating UV-A/blue light induction of *AtCHS* promoter activity. Northern analysis using various cryptochrome mutant lines, including a *cry1/cry2* double mutant, supports this hypothesis and, furthermore, suggests that other photoreceptors, which are able to respond to UV-A/blue light, exist in *Arabidopsis*.

5.2 OVEREXPRESSION OF CRY1 ENHANCES UV-A/Blue LIGHT INDUCED *AtCHS* PROMOTER ACTIVITY

The transient expression system was used to perform a gain of function experiment designed to investigate the role of CRY1 in the regulation of *AtCHS* promoter activity. To do this, protoplasts were co-transfected with a given *AtCHS* promoter: GUS reporter fusion construct and a CaMV 35S promoter: *CRY1* coding sequence fusion construct to over-express the CRY1 protein. Transfections with only the given *AtCHS* promoter: GUS reporter fusion construct were also performed and, as a control,

protoplasts transfected with the CaMV 35S: GUS reporter alone and with the CRY1 over-expression construct were assayed. The constructs used in this analysis are shown in Figure 5.1. The results of these transient expression assays are shown in Figure 5.2.

CRY1 over-expression has no effect on expression from the constitutive CaMV 35S promoter under any of the light conditions tested. However, CRY1 over-expression enhances expression from all of the *AtCHS* promoter constructs tested under high fluence rate white light and UV-A/blue light. Interestingly, no enhancement of *AtCHS* promoter activity by CRY1 was observed under UV-B light. Expression from the full-length *AtCHS* promoter (pAtCHS -1972) was increased by CRY1 over-expression by approximately 30 % under HWL conditions and more strikingly increased by approximately 80 % under UV-A/blue light conditions. This enhancement of *AtCHS* promoter activity by CRY1 is also observed with the minimal promoter (pAtCHS -164) and the tetramer promoter (pAtCHS LRU-4) which shows that the minimal promoter is sufficient to mediate this response. Furthermore, the full-length *AtCHS* promoter containing ACE and MRE block mutations showed similar enhancement with CRY1 over-expression. This suggests that this response is not specific to any particular region of the promoter. These observations indicate that CRY1 plays an important role in the UV-A/blue light regulation of *AtCHS* promoter activity in *At-glw* protoplasts. Furthermore, CRY1 does not appear to be involved in the UV-B light induction of expression from the *AtCHS* promoter.

**Figure 5.1 Schematic representation of constructs for analysis of
CRY1 overexpression on light regulation of *AtCHS*
promoter activity**

A. The *CaMV 35S* promoter, represented by a shaded region, shown in this plasmid is fused to the *CRY1* coding sequence, represented by a striped region, and has a nopaline synthase gene 3' untranslated sequence downstream of the coding sequence, represented by a second shaded region. This construct constitutively drives high levels of expression from the *CRY1* coding sequence under all light conditions.

B. The *AtCHS* promoter constructs shown are fused to the *uidA* open reading frame encoding GUS. The fusions have a common nopaline synthase gene 3' untranslated sequence downstream of the coding sequence (not shown). The ACE and MRE sequence elements of the LRU are indicated as a shaded box and a striped box respectively and block mutations in any of the sequence elements are represented by a shaded jagged circle.

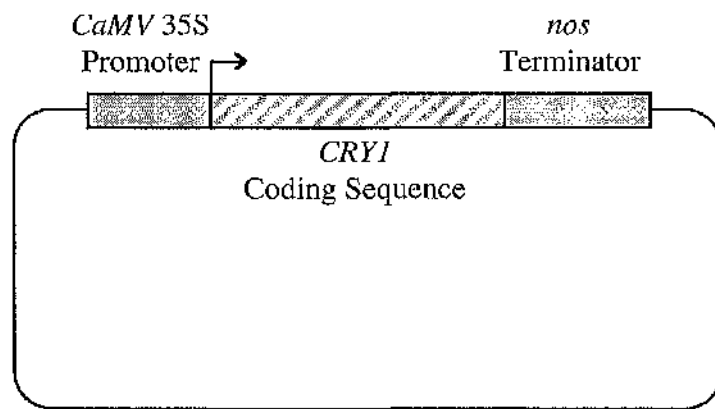
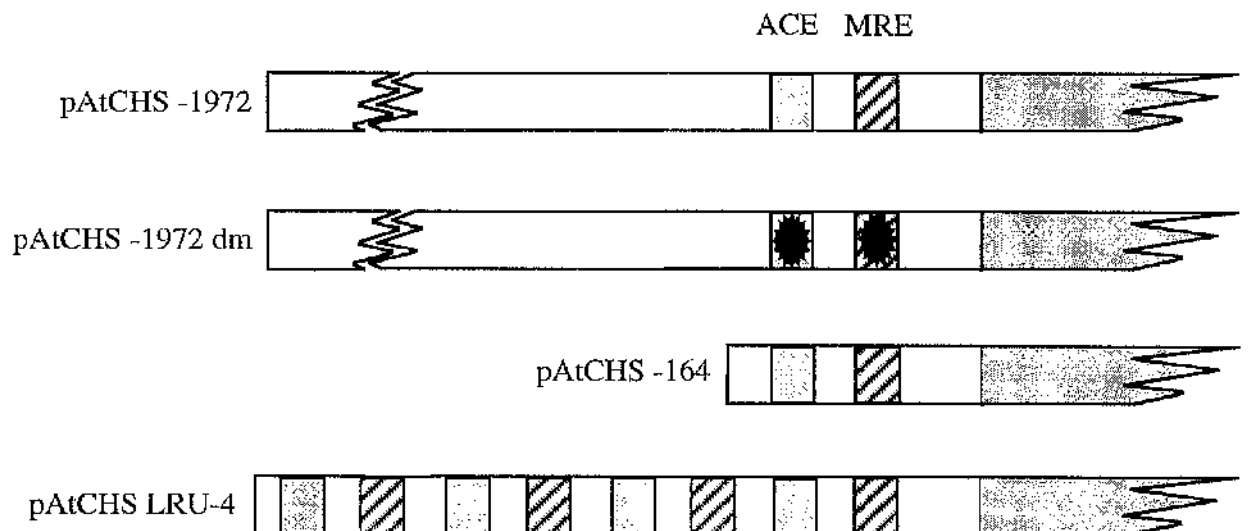
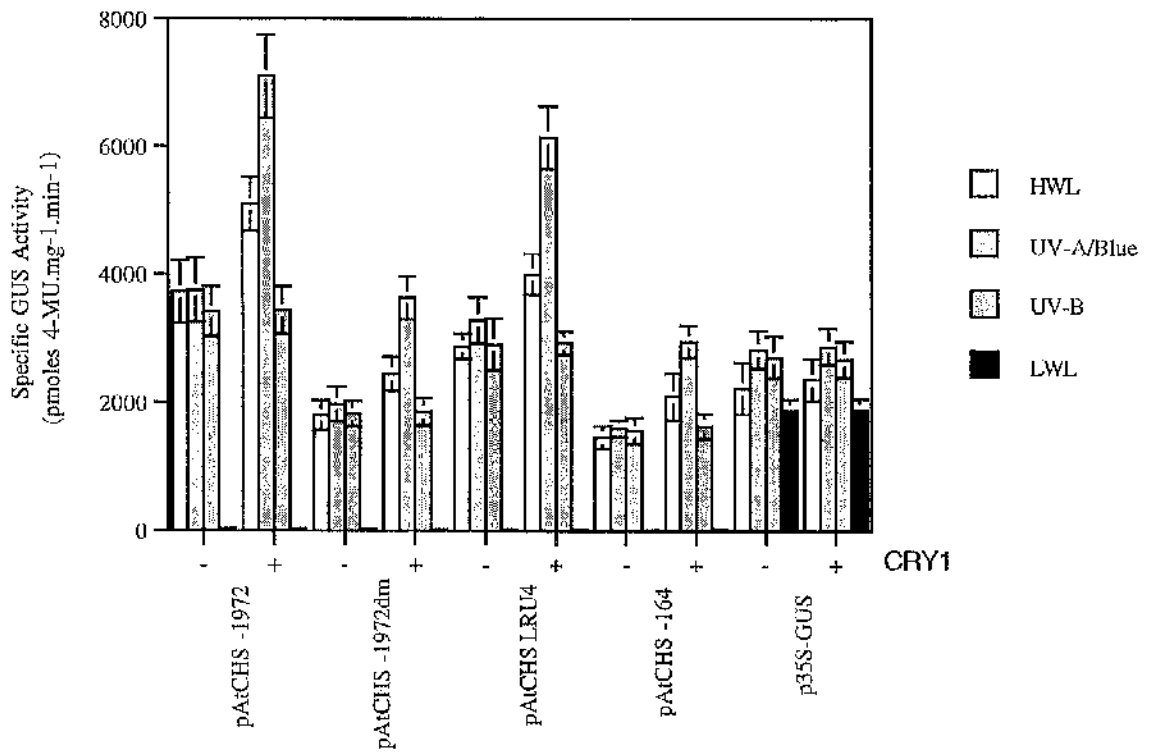
A**B**

Figure 5.2 Transient expression analysis of the effect of CRY1 overexpression on the light regulation of *AtCHS* activity in *At-glw* protoplasts

At-glw protoplasts were transfected with 20 μg of the indicated construct only (-) or 20 μg of the indicated construct plus 20 μg of the CRY1 overexpressing plasmid (+) and transferred to HWL ($100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light), UV-A/Blue ($80 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), UV-B ($3 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), Red ($80 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) or LWL ($20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light) and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



5.3 ANALYSIS OF *AtCHS* PROMOTER ACTIVITY IN TRANSIENT EXPRESSION ASSAYS USING *hy4* PROTOPLASTS

5.3.1 Transient Expression Assays Using Protoplasts Derived from *hy4* Cell Culture

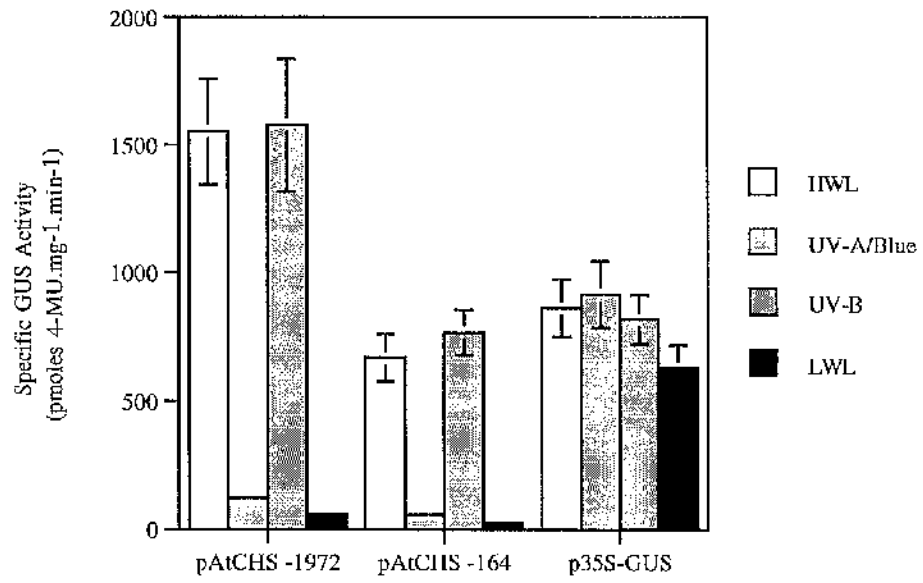
In order to further investigate the role of CRY1 in the control of *AtCHS* promoter activity we developed a transient expression system using protoplasts prepared from a *hy4* cell suspension culture. This cell culture was derived from a null mutant line (*hy4-2.23N*) of *Arabidopsis* lacking the CRY1 photoreceptor and has been previously described by Christie (1996). Initial attempts to prepare viable protoplasts from this cell culture proved unsuccessful. However, optimisation of growth conditions for suspension cultured *hy4* cells and reduction of cell clumps using a scalpel blade under aseptic conditions prior to digestion with cell wall degrading enzymes, improved protoplast production. Protoplast yield from *hy4* cells was low, 200 ml of cell culture routinely yielded approximately 2.5×10^6 protoplasts. However, FDA staining showed viability in excess of 98 % for these protoplasts (data not shown). *hy4* protoplasts were used to perform transient expression assays in the same manner as *At-glw* and *At-7* protoplasts were used previously.

5.3.2 UV-A/Blue Light Regulation of *AtCHS* Promoter Activity is Impaired in *hy4* Protoplasts

Transient expression assays were performed using *hy4* protoplasts in order to investigate the regulation of *AtCHS* promoter activity in the absence of CRY1. *hy4* protoplasts were transfected with full-length or minimal *AtCHS* promoter constructs or with the control CaMV 35S promoter construct, incubated under different light qualities and assayed for reporter gene activity. The results of this analysis are shown in Figure 5.3.

Figure 5.3 Transient expression analysis of light regulation of *AtCHS* promoter activity in *hy4* protoplasts

hy4 protoplasts were isolated and transfected with 20 μg of the construct indicated and transferred to HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light), UV-A/Blue ($80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$), UV-B ($3 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) or LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for three experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



Expression from the control 35S promoter was largely unaffected by the different light conditions. As in previous analyses, using both *At-glw* and *At-7* protoplasts, no *AtCHS* promoter activity was seen in *hy4* protoplasts incubated under non-inductive low fluence rate white light (LWL) conditions. However, light induced *AtCHS* promoter activity was observed from the full-length and minimal promoter constructs in protoplasts incubated under HWL or UV-B light. Only very low levels of reporter activity from both full-length and minimal promoters were seen in protoplasts incubated under UV-A/blue light. However, these levels of expression are significantly above those for protoplasts under non-inductive light conditions suggesting that there is some UV-A/blue light induction of *AtCHS* promoter activity in the absence of CRY1. These observations support the hypothesis that CRY1 is the primary photoreceptor mediating the UV-A/blue light induction of *AtCHS* promoter activity. However, low levels of *AtCHS* promoter activity in *hy4* protoplasts under UV-A/blue light suggest that at least one other photoreceptor can also mediate this response, albeit to a much lesser extent.

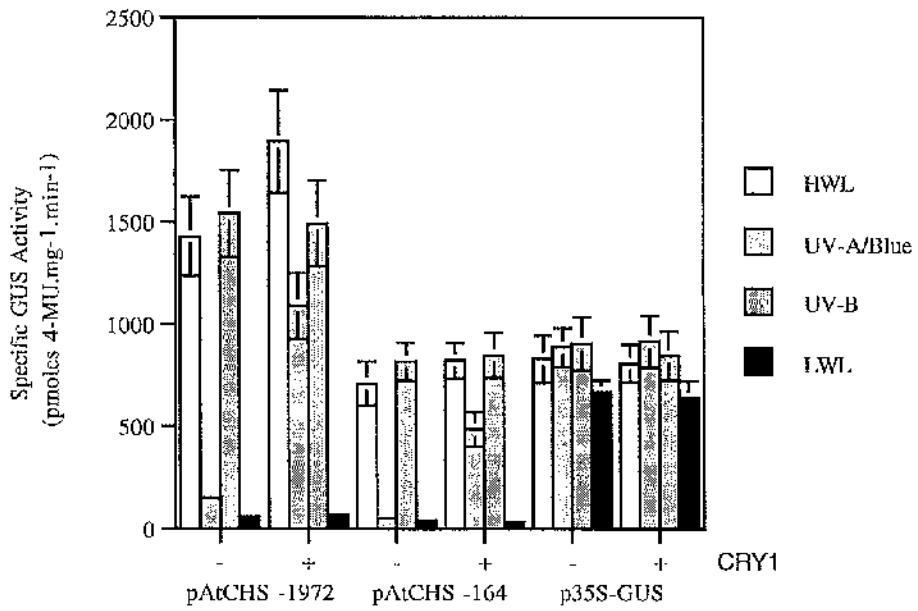
5.3.3 CRY1 Overexpression Restores UV-A/Blue Light Regulation of *AtCHS* Promoter Activity in *hy4* Protoplasts

We performed a gain of function experiment in order to confirm that the impaired UV-A/blue light induction of expression from the *AtCHS* promoter observed in 5.3.2 is, indeed, directly attributable to the absence of the CRY1 protein in *hy4* protoplasts. To do this, transient assays involving co-transfection of a construct over-expressing CRY1 with promoter: reporter fusions (as described in 5.2) were performed using *hy4* protoplasts. The results are shown in Figure 5.4.

Reporter activity from the CaMV 35S control promoter remained largely unaltered under all of the light conditions tested in the presence and absence of the over-expressing 35S: CRY1 construct. As in the previous analysis with *hy4* protoplasts,

Figure 5.4 Transient expression analysis of light regulation of *AtCHS* promoter activity in *hy4* protoplasts

hy4 protoplasts were isolated and transfected with 20 μg of the construct indicated and transferred to HWL ($100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light), UV-A/Blue ($80 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), UV-B ($3 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) or LWL ($20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light) and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for three experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



activity of the full-length *AtCHS* promoter (pAtCHS -1972) was induced by HWL and UV-B light but only low levels of promoter activity were detected in protoplasts under UV-A/blue light. However, when the CRY1 over-expressing construct was co-transfected with the full-length *AtCHS* promoter: GUS reporter fusion, then UV-A/blue light induced *AtCHS* promoter activity was partly restored. This result provides direct evidence that the CRY1 photoreceptor plays an important role in the UV-A/blue light regulation of *AtCHS* promoter activity in *Arabidopsis* protoplasts. In *hy4* protoplasts, as in *At-glw* protoplasts, CRY1 over-expression did not enhance UV-B light induced *AtCHS* promoter activity. This observation supports previous results suggesting that CRY1 is not involved in UV-B light induction of expression from the *AtCHS* promoter. The minimal *AtCHS* promoter also showed restored UV-A/blue light induction by CRY1 over-expression in *hy4* protoplasts. This observation supports the previous results which suggest that the minimal promoter is sufficient to mediate UV-A/blue light induction of *AtCHS* promoter activity via the CRY1 photoreceptor.

5.4. PRODUCTION OF A CRYPTOCHROME DOUBLE MUTANT

In order to further investigate the role of cryptochromes in the UV-A/blue light regulation of *AtCHS* expression, we produced a *fha1/hy4* double mutant line. It was thought that such a line would perhaps reveal responses mediated by cryptochrome which are not defective in *fha1* or *hy4* single mutant lines because of a functional overlap between the CRY2 and CRY1 proteins. The double mutant line was made by crossing *fha1.1* and *hy4-2.23N* plants. Pollen from *hy4* plants was used to fertilise the stamens of *fha1* before self-pollination could occur. Fertilised flowers were left in isolation to produce seed. The f1 seed were then grown, allowed to self-fertilise and the resulting seed collected. The f2 seed were sown, grown under blue light (30 $\mu\text{moles.m}^{-2}.\text{s}^{-1}$) and screened for long hypocotyls in order to select plants homozygous

for *hy4*. Seedlings were transferred to low fluence rate white light, grown until flowering and screened for a late flowering phenotype in order to select plants homozygous for *fha1*. Seeds collected from plants were used for the experiments described in 5.4.1, 5.5.1 and 5.5.2.

5.4.1 Phenotype of the Double Mutant Line

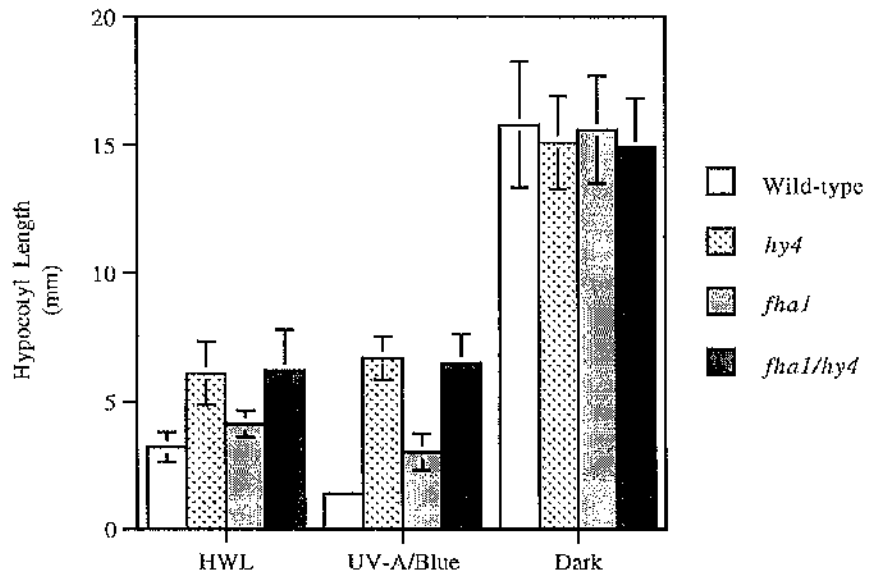
Before investigating the light regulation of *AtCHS* in *fha1/hy4* mutants, we characterised these new mutants by analysing the morphology of seedlings grown under different light conditions and studying the flowering times. The reasons for doing this were to define the phenotype of this new double mutant line and provide evidence that these plants are, indeed, *fha1/hy4* mutants.

5.4.1.1 Inhibition of Hypocotyl Elongation is Impaired in *fha1/hy4* Seedlings

The first analysis was the measurement of hypocotyl lengths of 8 day old seedlings grown under different light conditions. The results are shown in Figure 5.5. Seedlings of all of the genotypes tested showed very similar hypocotyl lengths when grown in darkness. Under high fluence rate white light, however, wild-type seedlings showed considerable inhibition of hypocotyl elongation. This response was impaired in *hy4* mutant seedlings where hypocotyl lengths were significantly longer than wild-type. This observation indicates that CRY1 is involved in this response to HWL. The hypocotyls of *fha1* mutant seedlings were slightly longer than those of wild-type seedlings under HWL. This shows that inhibition of the hypocotyl elongation response is only slightly impaired in these mutants under these light conditions which implies that CRY2 plays only a minor role in this response. The *fha1/hy4* double mutant seedlings showed hypocotyl lengths very similar to *hy4* seedlings and significantly longer than wild-type. This suggests that these seedlings are, indeed, homozygous for

Figure 5.5 Hypocotyl extension of cryptochrome mutants

Hypocotyl extension of wild type, *hy4*, *fha1* and *fha1/hy4* *Arabidopsis* seedlings grown in HWL ($100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light), UV-A/Blue ($30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) or darkness for 8 days are presented. The values shown are the means for 20 seedlings. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



the *hy4* mutation. Furthermore, given that these seedlings are *fha1/hy4* double mutants, these observations suggest that CRY1 plays a much more prominent role than CRY2 in the inhibition of hypocotyl elongation in response to HWL.

A similar pattern was observed when seedlings were grown under $30 \mu\text{moles}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ UV-A/blue light. Wild-type seedlings exhibited inhibition of hypocotyl elongation and had very short hypocotyls with a mean length of 1.4 mm. In contrast, *hy4* mutant seedlings were impaired in the inhibition of hypocotyl elongation. These seedlings exhibited long hypocotyls (6.9 mm) when grown in UV-A/blue light. This suggests that the CRY1 photoreceptor plays an important role in the UV-A/blue light mediated inhibition of hypocotyl elongation. In *fha1* seedlings, the inhibition of hypocotyl elongation was also impaired but to a much lesser extent than in *hy4* seedlings. *fha1* mutants had a mean hypocotyl length under UV-A/blue light which was double that of wild-type seedlings but only approximately 40 % of that of *hy4* seedlings. This suggests that CRY2 is involved in UV-A/blue light mediated inhibition of hypocotyl elongation at the fluence rate tested. However, the role of CRY2 does not appear to be as great as that of CRY1. In *fha1/hy4* seedlings, impaired inhibition of hypocotyl elongation was observed under UV-A/blue light. The mean hypocotyl length of *fha1/hy4* seedlings was very similar to that of *hy4* mutants under these light conditions. This observation supports the idea that CRY1 is important in mediating the inhibition of hypocotyl elongation in response to UV-A/blue light and CRY2 is involved to a lesser extent at the fluence rate tested. It is interesting that in the double mutant line, when both CRY1 and CRY2 are thought to be deficient, that the effect of these mutations was not additive and did not produce a phenotype more severe than the *hy4* mutation alone. This could be explained by a model where CRY1 is the primary photoreceptor mediating this response to UV-A/blue light. If the CRY2 photoreceptor mediates its effect by feeding a signal into the main CRY1 signal transduction pathway, then we would not necessarily expect to see any effect of knocking out the CRY2 photoreceptor when the CRY1 photoreceptor is already absent. Such a model is

consistent with *hy4* and *fha1/hy4* mutant lines exhibiting a similar phenotype with regard to this particular response. It is also worth noting that *fha1/hy4* double mutant seedlings grown under UV-A/blue light show hypocotyls approximately 50 % as long as their dark grown siblings. It is likely that this is due to the action of phytochromes which can absorb blue light, albeit to a much lesser extent than red or far-red light. It is, perhaps, this phytochrome mediated partial inhibition of hypocotyl elongation in response to UV-A/blue light that explains this observation.

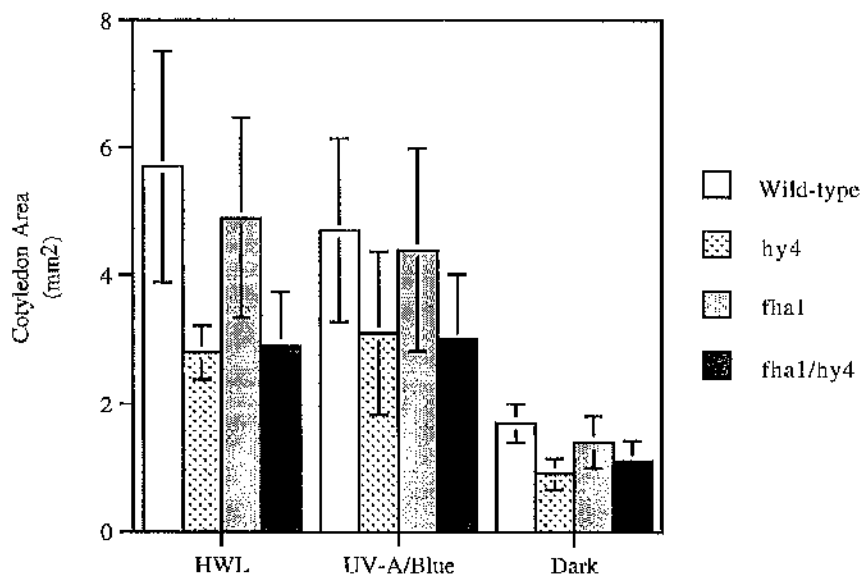
5.4.1.2 Cotyledon Expansion is Impaired in *fha1/hy4* Seedlings

Measurements were made of the cotyledon areas of the cryptochrome mutant lines. The results of this analysis are shown in Figure 5.6. All of the seedlings tested exhibited very little cotyledon expansion when grown in darkness. The cotyledon area of wild-type seedlings was found, on average, to be 5.7 mm² when grown under high fluence rate white light. *hy4* mutant seedlings showed impaired cotyledon expansion under these conditions and had a cotyledon area approximately half that of wild-type. *fha1* mutant seedlings also showed impaired cotyledon expansion under these conditions but to a much lesser extent. The mean cotyledon area of *fha1* mutants under HWL was approximately 80 % of wild-type. In *fha1/hy4* double mutant seedlings, the expansion of cotyledons was significantly impaired in HWL. The cotyledon areas of these seedlings were very similar to those observed with *hy4* mutants and were approximately 50 % of the mean wild-type cotyledon area. These observations suggest that both CRY1 and CRY2 are involved in cotyledon expansion in response to HWL. It appears that CRY1 plays a more prominent role than CRY2 in this response given that *hy4* seedlings exhibited a more severe phenotype than *fha1* mutants.

A similar pattern was observed when seedlings were grown under UV-A/blue light. *hy4* mutant seedlings showed significantly impaired cotyledon expansion when grown

Figure 5.6 Cotyledon area of cryptochrome mutants

Cotyledon area of wild type, *hy4*, *fha1* and *fha1/hy4* *Arabidopsis* seedlings grown in HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light), UV-A/Blue ($30 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) or darkness for 7 days are presented. The values shown are the means for 20 seedlings. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



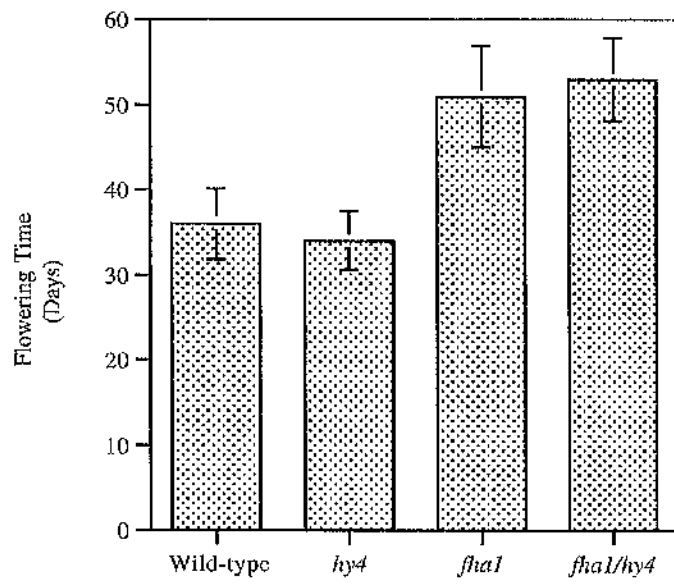
under 30 $\mu\text{moles.m}^{-2}.\text{s}^{-1}$ UV-A/blue light. The mean cotyledon area of *hy4* mutants was less than 70 % of wild-type seedlings. Under the same light conditions, *fha1* mutant seedlings showed very little impairment of cotyledon expansion with a mean cotyledon area approximately 93% of wild-type. The *fha1/hy4* double mutant line exhibited impaired cotyledon expansion under UV-A/blue light. As in HWL conditions, *fha1/hy4* seedlings showed similar cotyledon areas to those observed with *hy4* mutant seedlings, approximately 70 % of the mean wild-type area. These results indicate that both CRY1 and CRY2 are involved in mediating this response but CRY1 plays a more important role under the light conditions tested. As previously observed with regard to the inhibition of hypocotyl elongation (5.4.2.1), the *fha1/hy4* double mutant line does not have a more severe phenotype for cotyledon expansion than the *hy4* line. Again, this is consistent with a model where CRY1 is the primary photoreceptor mediating the response and signals from the CRY2 photoreceptor feed into the CRY1 pathway to regulate the response.

5.4.1.3 *fha1/hy4* Mutants Exhibit a Late Flowering Phenotype

The mean flowering times of the cryptochrome mutant lines are shown in Figure 5.7. The mean flowering times of wild-type and *hy4* mutant lines when grown under constant low fluence rate white light are very similar at around 7 weeks. However, *fha1* mutants and *fha1/hy4* double mutants grown under that same conditions show mean flowering times much later (approximately 2 weeks) than that of wild-type plants. This is consistent with the findings of Guo *et al.* (1998) who had previously identified *fha1* as a late flowering mutant under long day conditions. The late flowering phenotype of the *fha1/hy4* double mutant line indicates that it is, indeed, homozygous for the *fha1* mutation. In addition, these observations suggest that the CRY2 photoreceptor plays an important role in the regulation of flowering time. Interestingly, this mutational analysis suggests that, at least under the conditions tested here, the CRY1 photoreceptor is not involved in this response.

Figure 5.7 Flowering time of cryptochrome mutants

The flowering time of wild type, *hy4*, *fha1* and *fha1/hy4* *Arabidopsis* seedlings grown in constant LWL ($20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ white light) are presented. The values shown are the mean flowering times for 20 seedlings. Error bars indicate the SD for each value.



5.5 ANALYSIS OF *AtCHS* REGULATION IN CRYPTOCHROME MUTANTS

5.5.1 Analysis of *AtCHS* Regulation in Cryptochrome Mutants

Transient expression analysis (described in 5.3.1 - 5.3.3) indicated that the CRY1 photoreceptor plays an important role in the photoregulation of *AtCHS* expression. Therefore, we wanted to further analyse this role and that of the CRY2 photoreceptor in *AtCHS* regulation. Northern blot analysis was used to investigate the effect of mutations in the genes encoding CRY1 and CRY2 on the control of *AtCHS* transcript levels. Northern blots were performed using four different *Arabidopsis* lines; wild type, *hy4* (a *cry1* mutant line), *fh1* (a *cry2* mutant line) and the *fh1/hy4* double mutant (a *cry1/cry2* mutant line) described in 5.4.1 and 5.4.2. Plants were grown for 21 days in a low fluence rate of white light ($20 \mu\text{moles.m}^{-2}.\text{s}^{-1}$) and then transferred to the light treatments indicated. Leaf tissue was harvested and used to measure *AtCHS* transcript levels. These experiments were performed 2-3 times and on each occasion a similar result was obtained. The results from one set of northern blots are shown in Figure 5.8.

Figure 5.8 A shows transcript levels from the *AtCHS* gene in leaves of wild type plants in response to different qualities of light. *AtCHS* transcripts could not be detected in leaves of plants grown under low fluence rate white light. *AtCHS* transcript levels are high following transfer for 6 h to high fluence rate white light, UV-A/blue light and UV-B light. These measurements are in agreement with the pattern of *AtCHS* regulation observed in protoplasts from the *At-glw* cell culture. This suggests that the transient expression assays accurately reflect levels of *AtCHS* transcription *in vivo*. These observations are also in agreement with previous northern analyses which have shown that *AtCHS* transcripts accumulate in leaf tissue in response to HWL, UV-A/blue and UV-B light (Feinbaum *et al.*, 1991; Jackson *et al.*, 1995; Fuglevand *et al.*, 1996).

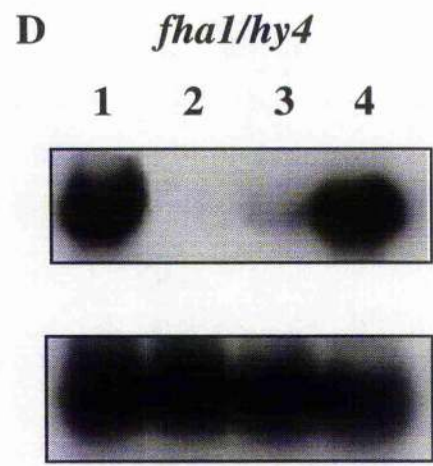
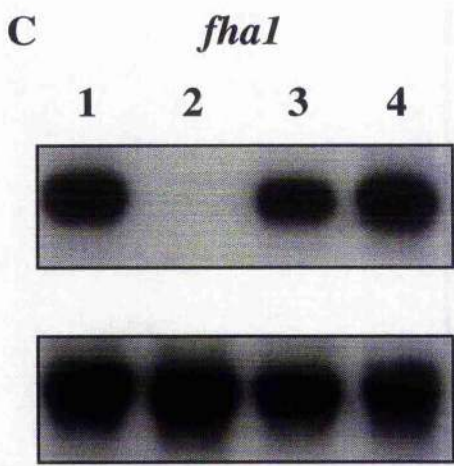
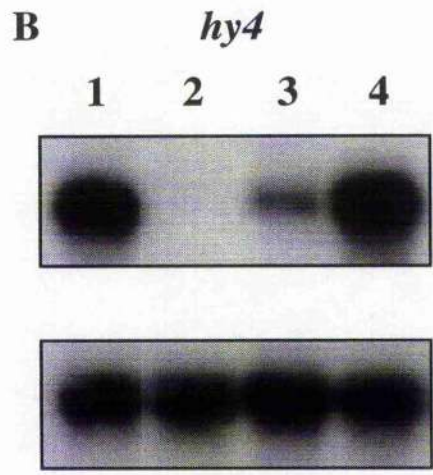
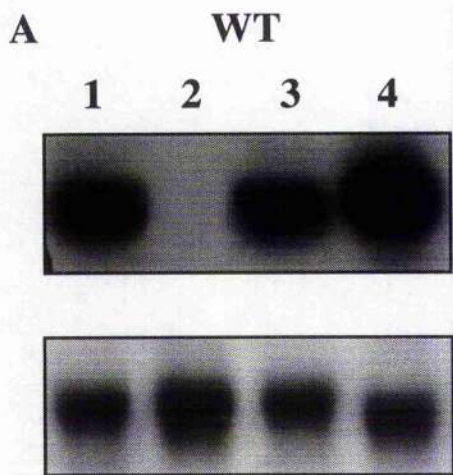
The results of northern analysis using *hy4* leaf tissue are shown in Figure 5.8 B. As with wild type tissue, LWL does not induce *AtCHS* transcription. There are high levels of *AtCHS* transcript accumulation in HWL and in UV-B light. However, transcript levels in tissue treated with UV-A/blue light are significantly reduced when compared with wild-type. Interestingly, though, there are still detectable levels of transcripts under these conditions. These observations are in agreement with previous transient expression studies (5.2 and 5.3) and northern analysis (Jackson *et al.*, 1995; Fuglevand *et al.*, 1996) and suggest that CRY1 is the primary photoreceptor involved in the regulation of *AtCHS*. The observation that *AtCHS* transcription is not abolished in *hy4* mutant tissue implies that one or more photoreceptor(s) other than CRY1 also play a role in the regulation of CRY1 by UV-A/blue light. It is noteworthy that transcript levels appear unaltered in UV-B treated *hy4* tissue. This indicates that *AtCHS* transcription in response to UV-B light is regulated by (an)other photoreceptor(s).

In *fha1* leaf tissue, the pattern of *AtCHS* transcript accumulation is very similar to that of wild type tissue (see Figure 5.8 C). No detectable levels of *AtCHS* transcript accumulation were found in tissue grown in LWL. High levels of transcripts were detected in *fha1* leaf tissue treated with HWL, UV-A/blue and UV-B. In *fha1* mutants, there was no significant alteration in transcript levels under UV-A/blue light compared to wild-type tissue. This suggests that, at least at the fluence rates tested, CRY2 does not play a major role in the regulation of *AtCHS* transcription by UV-A/blue light. Similarly, as with *hy4* tissue, UV-B induced transcript accumulation appeared unaltered in *fha1* mutants suggesting that (an)other photoreceptor(s) is involved in UV-B regulation.

Figure 5.8 D shows the measurement of transcript levels in *fha1/hy4* leaf tissue. As with all previous analyses, no detectable levels of *AtCHS* transcripts were found under LWL conditions. High levels of transcript accumulation were observed in HWL and

**Figure 5.8 *AtCHS* induction in response to different qualities of light
in cryptochrome mutants**

Arabidopsis plants grown in LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) for 21 days were transferred to the following light qualities for 6 h: $100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light (lane 1), $20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light (lane 2), $80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ UV-A/blue light (lane 3) or $3 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ UV-B light (lane 4). RNA was isolated from leaves, $20 \mu\text{g}$ separated on a 1.3 % denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and autoradiographed. The membrane was stripped of radioactivity and reprobbed with a *Phaseolus vulgaris H1* probe. This analysis was performed with wild type (A), *hy4* (B), *fha1* (C) and *fha1/hy4* (D) plants.



UV-B conditions. UV-A/blue induction of transcripts was greatly impaired, although a signal was still detectable, in *fha1/hy4* tissue. This suggests that other, as yet undefined, photoreceptors can mediate UV-A/blue light induction of *AtCHS* transcription albeit at very low levels. As with the single mutants (*hy4* and *fha1*), regulation of *AtCHS* transcript accumulation appears unaltered in UV-B light, confirming that (an)other photoreceptor(s) is involved in mediating this response.

5.5.2 Analysis of Simultaneous UV-B and UV-A/blue Light on *AtCHS* Regulation in Cryptochrome Mutants

In 1996, Fuglevand *et al.* reported that the level of *CHS* transcription is stimulated by an interaction between UV and blue light signalling pathways. It was demonstrated that the response to UV-B is enhanced by synergistic interactions with separate UV-A and blue light signalling pathways that serve to maximise the level of *CHS* expression. The authors showed that the level of *CHS* promoter: GUS expression in transgenic *Arabidopsis* was much greater in plants exposed to UV-B and blue or UV-B and UV-A light together than in plants given either light quality alone. The 4- to 8-fold increase was more than additive and indicated a synergistic interaction between separate phototransduction pathways. We decided to investigate this response in cryptochrome mutants in order to further analyse the roles of CRY1 and CRY2 in the photoregulation of *AtCHS*. Northern analysis was used to measure *AtCHS* transcript levels for this study. Experiments were performed 2-3 times and on each occasion a similar result was obtained. The results from one set of experiments are shown in Figure 5.9.

Figure 5.9 A shows the measurement of transcript levels in wild type leaf tissue. As in the previous analysis, no *AtCHS* transcript accumulation is seen in tissue grown in low fluence rate white light. Light induced *AtCHS* transcript accumulation is evident in UV-A/blue light and in UV-B light. In plants exposed to both UV-A/blue and UV-B, the accumulation of transcripts is much greater than observed for either light quality alone.

The levels of transcripts in these plants appears to be much greater than the sum of the transcript levels in UV-A/blue and UV-B light. These data provide evidence of the synergistic interaction between the UV-A/blue and UV-B signal transduction pathways described above.

The northern analysis of *AtCHS* expression in *hy4* leaf tissue is shown in Figure 5.9 B. As in wild type tissue, no detectable *AtCHS* expression is induced by low fluence rate white light. There appears to be no *AtCHS* transcript accumulation in *hy4* tissue treated with UV-A/blue light. However, a weak signal can be detected when the autoradiograph is overexposed (data not shown). This is in agreement with previous northern analysis (Figure 5.8 B) which shows that only very low levels of *AtCHS* transcripts are present in *hy4* leaf tissue treated with UV-A/blue light. UV-B induced transcript accumulation appears to be unaltered in *hy4* tissue when compared with wild-type. In tissue treated with both UV-A/blue and UV-B light, very high levels of *AtCHS* transcripts were measured and this response also appears to be unaltered when compared with wild type. These observations suggest that the CRY1 photoreceptor is not involved in mediating the synergistic induction of *AtCHS* transcription. This is in agreement with the findings of Fuglevand *et al.* (1996).

In *fha1* tissue, as in the previous *fha1* northern analysis, the pattern of *AtCHS* transcript accumulation is very similar to that of wild type under the light conditions tested (Figure 5.9 C). No induction of *AtCHS* transcripts is seen in LWL. Expression of *AtCHS* appears to be largely unaltered when compared to wild type under UV-A/blue light and UV-B light. Interestingly, as seen in *hy4* tissue, the synergistic induction of *AtCHS* transcription by UV-A/blue and UV-B light appears to be unaltered in *fha1* mutants when compared to wild type. These results suggest that CRY2 is not involved in the synergistic induction of *AtCHS* transcript accumulation.

Figure 5.9 D shows the measurement of transcript levels in the *fha1/hy4* mutant line. No induction of *AtCHS* transcripts is detectable in leaf tissue treated with low fluence rate white light. As shown in Figure 5.8 D, there appears to be no *AtCHS* transcript accumulation in *fha1/hy4* tissue treated with UV-A/blue light. However, a very weak signal can be detected when the autoradiograph is overexposed (data not shown). UV-B light induction of *AtCHS* transcription appears to be unimpaired in *fha1/hy4* mutant tissue. Interestingly, high levels of *AtCHS* transcripts were detected in tissue treated with UV-A/blue and UV-B light, indicating that the synergistic responses are not altered in *fha1/hy4* mutants. These observations indicate that neither CRY1 nor CRY2 is involved in mediating the UV-A/blue and UV-B synergistic induction of *AtCHS* transcription. This is consistent with the single mutant results which suggest that neither CRY1 nor CRY2 mediate the synergistic responses. Thus, the unaltered synergistic responses in *fha1* or *hy4* single mutants was not due to functional overlap of the CRY1 and CRY2 photoreceptors. These results also indicate that the synergistic responses are mediated by (a) photoreceptor(s) other than CRY1 and CRY2. This implies that another photoreceptor exists which mediates responses to UV-A/blue light other than the two previously defined cryptochromes.

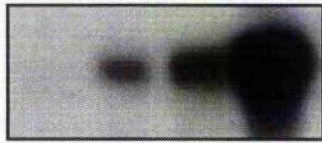
5.6 DISCUSSION

The flexibility of the transient expression system allowed us to analyse the role of the CRY1 photoreceptor in the regulation of *AtCHS* promoter activity. Co-transfection assays enabled us to over-express CRY1 and analyse the effect on regulation from the *AtCHS* promoter. This study indicated that CRY1 is involved in the UV-A/blue light regulation of *AtCHS* but not in regulation by UV-B light. The value of this gain of function assay, in contrast to measurements with *cry1* mutant plants, is that functional

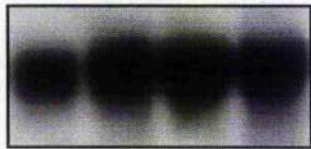
Figure 5.9 Effects of a simultaneous UV-B and UV-A/blue light treatment on *AtCHS* transcript accumulation in cryptochrome mutants

Arabidopsis plants grown in LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) for 21 days were transferred to the following light qualities for 6 h: $20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light (lane 1), $100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light (lane 2), $80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ UV-A/blue light (lane 3) or $3 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ UV-B light plus $80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ UV-A/blue light (lane 4). RNA was isolated from leaves, $20 \mu\text{g}$ separated on a 1.3 % denaturing agarose gel and blotted to a nylon membrane. The northern blot was probed with an *Arabidopsis CHS* probe, washed and autoradiographed. The membrane was stripped of radioactivity and reprobbed with a *Phaseolus vulgaris H1* probe. This analysis was performed with wild type (A), *hy4* (B), *fha1* (C) and *fha1/hy4* (D) plants.

A **WT**
1 2 3 4

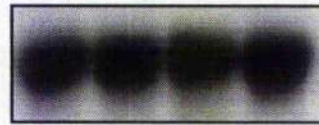
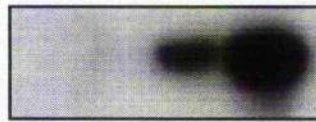


CHS

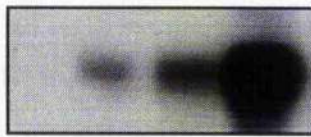


HI

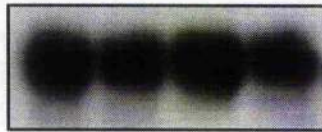
B *hy4*
1 2 3 4



C *fha1*
1 2 3 4

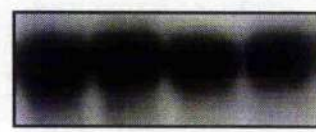


CHS



HI

D *fha1/hy4*
1 2 3 4



redundancy of cryptochromes cannot confuse the interpretation. This work therefore extends, and complements, previous measurements of transcript levels in *hy4* (Jackson and Jenkins, 1995; Funglevand *et al.*, 1996). Experiments involving co-transfection of 35S-CRY2 fusions should now be undertaken to examine the role of CRY2 in the UV-A/blue and UV-B induction of *AtCHS* transcription.

Use of a *hy4* cell culture to produce protoplasts for transient assays allowed us to study the effect of CRY1 on *AtCHS* promoter activity in a *cry1* null background. These analyses suggested that CRY1 is the primary photoreceptor mediating UV-A/blue light regulation of *AtCHS* expression. However, it remains possible that other UV-A/blue light photoreceptor(s) can also induce expression from the *AtCHS* promoter albeit at a much lower level. This system could now be used to undertake structure-function analyses of the CRY1 protein.

Production of a novel *cry1/cry2* mutant line and studies using existing *cry1* and *cry2* mutants allowed us to examine the role of cryptochromes in several UV-A/blue mediated responses. These studies suggested that, in *Arabidopsis*, CRY1 is the primary UV-A/blue light photoreceptor involved in the inhibition of hypocotyl elongation and in the control of cotyledon expansion. CRY2 is clearly involved in mediating these responses but to a much lesser extent than CRY1 at the fluence rates tested. Lin *et al.* (1998) demonstrated that CRY2 is most effective at low fluence rates of blue light because, in contrast to CRY1, the photoreceptor is degraded at high fluence rates of blue light. This observation perhaps explains why, in this study, CRY2 appears to play a minor role in these responses. However, CRY2 does play an important role in the regulation of flowering time, a response in which CRY1, at least under the conditions tested in this study, appears to be uninvolved.

Northern analyses using these cryptochrome mutant lines supported the hypothesis that CRY1 is the primary photoreceptor mediating UV-A/blue light regulation of *AtCHS*

transcription. The *cry2* (*fha1*) mutant was unaltered in *AtCHS* transcript levels in UV-A/blue light compared to wild type at the fluence rates employed. Furthermore, neither CRY1 nor CRY2 are involved in the UV-B light regulation of *AtCHS* expression. This is a significant observation, because information had only previously been reported for the *cry1* (*hy4*) mutant (Fuglevand *et al.*, 1996). Although *hy4* retained UV-B induction of *AtCHS* transcripts, it was possible that this was due to the action of CRY2. However, the experiments in this study with the *fha1/hy4* double mutant refute this possibility.

Interestingly, the synergistic induction of *AtCHS* transcription by UV-A/blue and UV-B light was unaltered in the *cry1/cry2* double mutant. This observation indicates that (an)other photoreceptor(s) is responsible for mediating the UV-A/blue light signal for this response, thus providing evidence for the existence of at least one other, as yet uncharacterised, UV-A/blue light photoreceptor. It should be noted that in previous experiments UV-A and blue light were each shown to mediate a synergistic response with UV-B light, whereas in the present study UV-A and blue light were not used separately. This maximises the extent of the synergism but has the disadvantage that the two separate synergistic interactions cannot be studied. Thus, further experiments should be undertaken with the double mutant to see whether the blue and UV-B and UV-A and UV-B synergistic interactions are both unaltered. Nevertheless, the fact that the level of transcripts in the double mutant in UV-A/blue and UV-B light is indistinguishable from that in wild type, suggests that each synergism is unimpaired.

CHAPTER 6: THE ROLE OF SUGAR SENSING IN THE CONTROL OF *AtCHS* EXPRESSION

6.1 INTRODUCTION

Physiological, biochemical and molecular studies have demonstrated that various plant genes are subject to regulation by sugars. A well established example is that the genes encoding proteins involved in photosynthesis are subject to negative feedback regulation by sugars accumulated through photosynthetic carbon fixation (reviewed by Graham, 1996; Sheen, 1994). For example, Sheen (1990) provided evidence that sugars directly repress photosynthetic gene expression using protoplasts from maize leaves in a transient expression system for analysis of promoter-reporter gene fusions. It was found that the transcriptional activity of seven different promoters from photosynthetic genes of maize was repressed by sucrose and glucose. Similar repression of photosynthetic genes has been demonstrated in several species, including *Arabidopsis*, where induction of the *rbcS* transcript by light was inhibited when plants were grown on media containing sucrose (Harter *et al.*, 1993).

However, metabolic regulation is not restricted to photosynthetic genes. Graham *et al.* (1994) reported that genes encoding the glyoxylate cycle enzymes, malate synthase (*MS*) and isocitrate lyase (*ICL*), of cucumber were subject to sugar repression. In addition the alpha-amylase genes *Amy3D* and *Amy3E* of rice and the sucrose synthase (*Sh1*) gene of maize are known to be repressed by sugars (Huang *et al.*, 1993; Maas *et al.*, 1990). In contrast, sugars have also been shown to stimulate gene expression. For example, genes encoding patatin in potato and β -amylase and sporamin in sweet potato have been reported to be induced by sucrose (Grierson *et al.*, 1994; Liu *et al.*, 1990; Ishiguro and Nakamura, 1992). *CHS* gene expression has also been shown to be stimulated by sucrose (Moalem-Beno *et al.*, 1997; Tsukaya *et al.*, 1991). Furthermore, Urwin and Jenkins (1997) demonstrated that sucrose, glucose or fructose was required

to stimulate *CHS* gene expression in *Phaseolus vulgaris* protoplasts. It is therefore evident that sugars have a central role in the metabolic regulation of gene expression.

We investigated the role of sugar regulation in the control of *AtCHS* gene expression using the *At-glw* protoplast transient expression system. Initial analysis revealed that there is a sucrose requirement for light-induced *AtCHS* promoter activity in *At-glw* protoplasts. Previous studies have shown that sugars other than sucrose can mediate regulation of gene expression (Graham, 1996; Sheen, 1994). Experiments with sugar analogues which are either non-metabolisable or partially metabolised, indicate that metabolism of sugars by hexokinase is necessary for regulation (Graham *et al.*, 1994; Jang and Sheen, 1994). Hexokinase mediates this regulation, as the name suggests, by phosphorylating hexose sugars. To determine whether a similar mechanism was operative in the protoplast transient expression system, we investigated whether glucose and fructose, the monosaccharide constituents of sucrose, 3-O-methylglucose (an analogue of glucose which is thought to be non-metabolisable) and 2-deoxyglucose (a glucose analogue which is thought to be phosphorylated by hexokinase but not further metabolised) were able to regulate *AtCHS* promoter activity. The results of this analysis suggest that the initial signal for sugar stimulation is consistent with the model of signalling by hexokinase phosphorylation of hexose sugars.

6.2 SUCROSE IS REQUIRED FOR *AtCHS* LIGHT REGULATION IN TRANSIENT EXPRESSION ASSAYS

In order to determine whether sucrose was required for light induction of *AtCHS* promoter activity, we performed transient expression assays were performed in which protoplasts were incubated in the presence and absence of 20 mM sucrose. Various *AtCHS* promoter constructs, described in Chapter 4, including the full-length (-1972 bp) and the minimal (-164 bp) *AtCHS* promoter-GUS reporter fusions were transfected

into At-glw protoplasts for this study. The transfected protoplasts were incubated for 16 h under high fluence rate white light or non-inductive low fluence rate white light in medium containing 400 mM mannitol or 20 mM sucrose and 380 mM mannitol. The results of this analysis are shown in Figure 6.1.

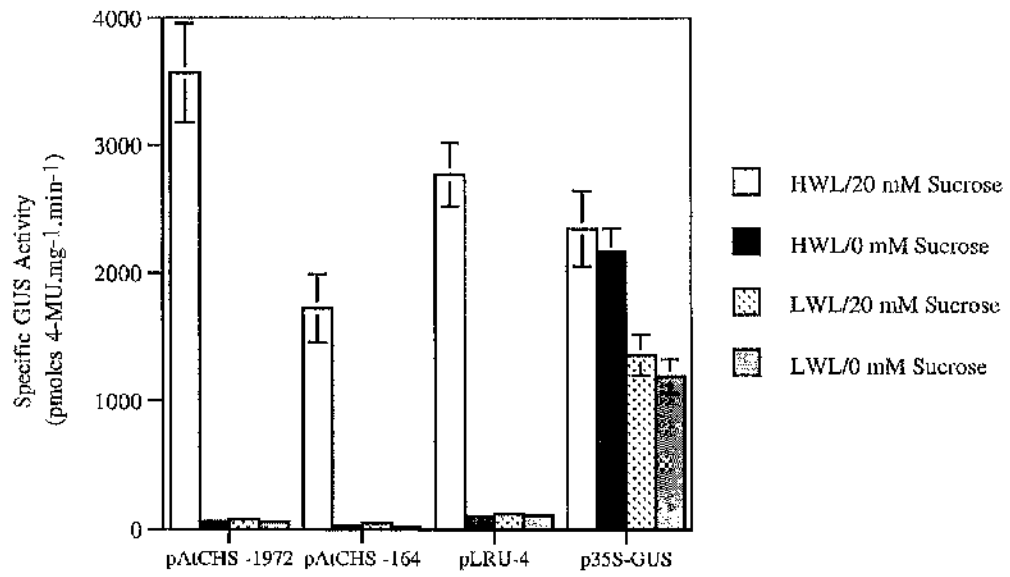
None of the *AtCHS* promoter constructs tested drove expression of the GUS reporter gene under low fluence rate light conditions in the presence or absence of sucrose. In the presence of 20 mM sucrose, protoplasts containing the full-length (p*AtCHS* -1972), minimal (p*AtCHS* -164) and tetramer (pLRU-4) constructs showed light induced reporter gene activity. Interestingly, light induced reporter gene activity was not seen in the absence of sucrose in protoplasts containing any of these constructs. These observations suggest that light induced *AtCHS* promoter activity is sugar-dependent in At-glw protoplasts. Furthermore, this effect is seen in the minimal and tetramer promoter containing only the LRU as well as in the full-length *AtCHS* promoter, which indicates that the minimal *AtCHS* promoter is sufficient to mediate this response.

6.3 0.5 mM SUCROSE IS SUFFICIENT FOR *AtCHS* LIGHT REGULATION

All of the transient expression assays performed up to this point had used concentrations of sucrose much higher than those found under physiological conditions. We decided to investigate how lower concentrations of sucrose affect the light regulation of expression from the *AtCHS* promoter. For this study, At-glw protoplasts were transfected with either the full-length *AtCHS* promoter: GUS reporter construct (p*AtCHS* -1972) or the constitutive CaMV 35S promoter: GUS reporter (p35S-GUS) construct. The protoplasts were then incubated under inductive or non-inductive light conditions in medium containing sequentially lower concentrations of

Figure 6.1 Sucrose is required for light induced *AtCHS* promoter activity in *At-glw* protoplasts

At-glw protoplasts were isolated and transfected with 20 μg of the indicated construct and transferred to HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) in 20 mM sucrose, HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) in the absence of sucrose, LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) in 20 mM sucrose or LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) in the absence of sucrose and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



sucrose. The osmotic conditions of the medium were maintained by ensuring a complementary concentration of mannitol was present. Mannitol is thought to be an inert sugar which is not metabolised. The results of this analysis are shown in Figure 6.2.

Whilst expression from the CaMV 35S promoter remains relatively unchanged in different sucrose concentrations, expression from the *AtCHS* promoter under high fluence rate white light decreases as the concentration of sucrose decreases. In 5 mM sucrose, light induced reporter activity is very similar to that in 10 and 20 mM sucrose. However, promoter activity is diminished in 1 mM sucrose to approximately 65 % of that in 20 mM sucrose. The *AtCHS* promoter is still light responsive in 0.5 mM sucrose although light induction of expression is greatly reduced under these conditions. No light induced *AtCHS* promoter activity was observed in the absence of sucrose. These results suggest that concentrations of sucrose as low as 0.5 mM are sufficient for light induced activity of the *AtCHS* promoter in *At-glw* protoplasts, although the fold induction is much reduced below 1 mM.

6.4 *AtCHS* IS LIGHT REGULATED IN THE PRESENCE OF GLUCOSE

In order to investigate whether other sugars, hexoses in particular, affect *AtCHS* promoter activity, transient expression assays were performed where *At-glw* protoplasts were incubated in different concentrations of glucose. These experiments were performed as described in 6.3 substituting glucose in place of sucrose. The results are shown in Figure 6.3.

Figure 6.2 Effect of decreasing the concentration of sucrose on *AtCHS* promoter activity in *At-glw* protoplasts

At-glw protoplasts were isolated and transfected with 20 μg of the indicated construct and transferred to HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) or LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) in medium containing decreasing concentrations of sucrose (20 mM to 0mM) as indicated and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.

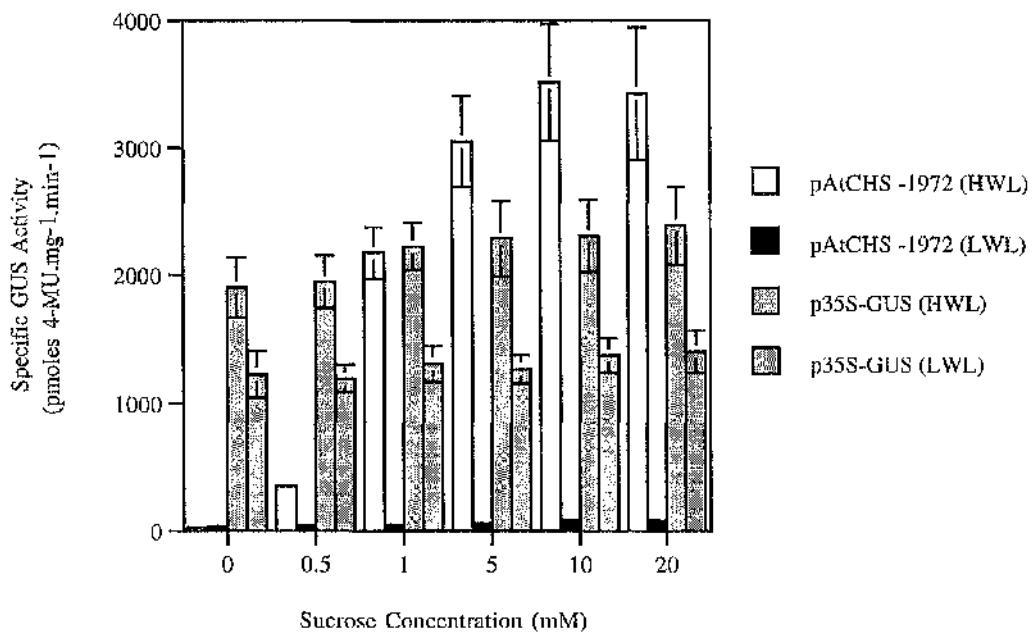
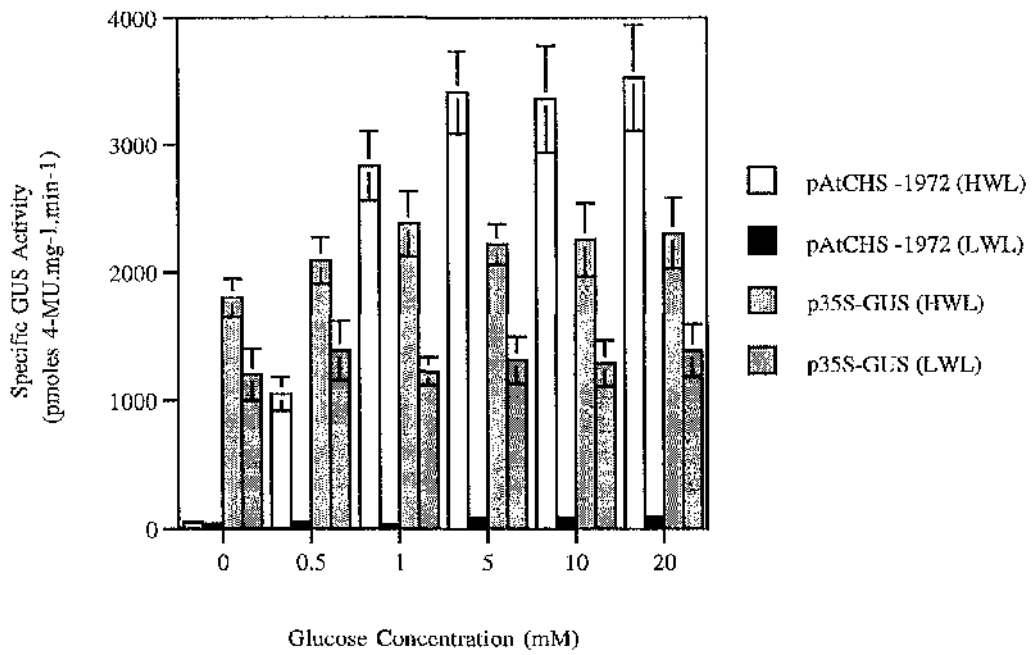


Figure 6.3 Effect of different concentrations of glucose on *AtCHS* promoter activity in *At-glw* protoplasts

Protoplasts were isolated from *At-glw* cells and transfected with 20 μg of the indicated construct and transferred to HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) or LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) in medium containing a range of different concentrations of glucose (20 mM to 0mM) as indicated and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



Expression from the CaMV 35S promoter remains relatively unchanged in different concentrations of glucose. Light induced *AtCHS* promoter activity was observed in all concentrations of glucose. In the absence of glucose, no light induction of the *AtCHS* promoter activity was detected. In 5, 10 and 20 mM glucose, high levels of light induced reporter gene activity were observed. *AtCHS* promoter activity was slightly diminished in 1 mM glucose and in 0.5 mM glucose only reached approximately 30 % of that seen in 20 mM glucose. These observations show that glucose is capable of mediating expression from the *AtCHS* promoter in *At*-glw protoplasts. Furthermore, light induced *AtCHS* promoter activity is greater in protoplasts in 5 mM glucose than those in 5 mM sucrose. This suggests that this sugar-mediated response is more sensitive to glucose than to sucrose.

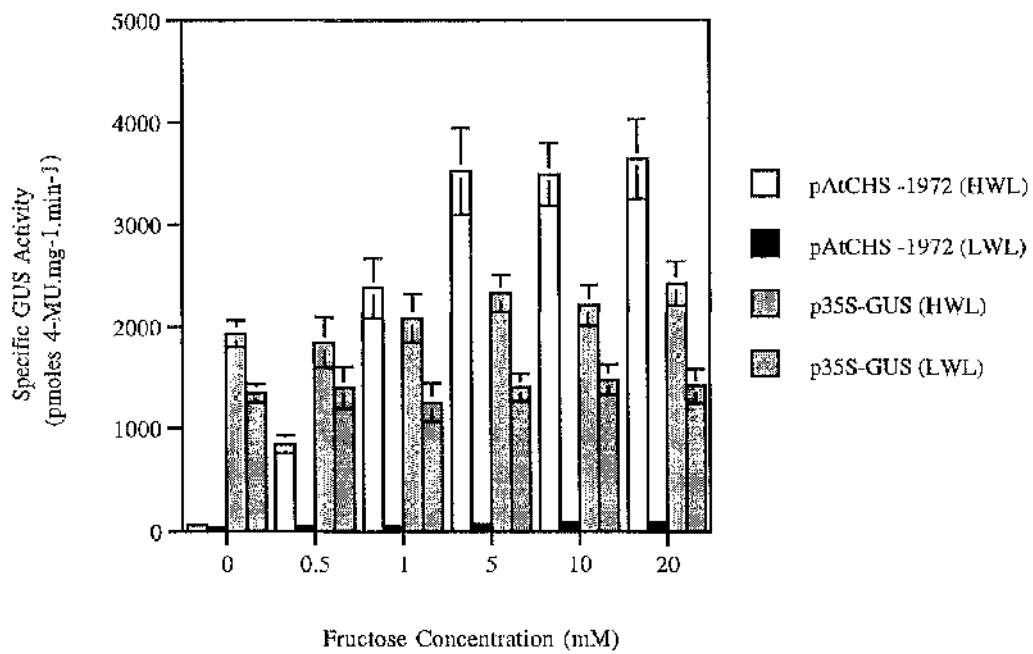
6.5 FRUCTOSE AFFECTS LIGHT INDUCED *AtCHS* PROMOTER ACTIVITY

Transient expression assays were performed to investigate the effect of different concentrations of fructose on *AtCHS* promoter activity. These assays were performed as described in 6.3 substituting fructose in place of sucrose. The results are shown in Figure 6.4.

As observed for sucrose and glucose, expression from the control CaMV 35S promoter remained largely unaffected by different concentrations of fructose. *AtCHS* promoter activity was observed under inductive light conditions in all concentrations of fructose tested but not in the absence of fructose. High levels of light induced *AtCHS* driven reporter activity were observed at higher concentrations (5, 10 and 20 mM) of fructose. Light induced reporter activity was slightly diminished in protoplasts in 1 mM fructose and considerably lower in 0.5 mM fructose, only reaching approximately 25 % of the

Figure 6.4 Effect of different concentrations of fructose on *AtCHS* promoter activity in *At-glw* protoplasts

At-glw protoplasts were isolated and transfected with 20 μg of the indicated construct and transferred to HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) or LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) in medium containing a range of different concentrations of fructose (0 mM to 20mM) as indicated and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



value observed for protoplasts in 20 mM fructose. These observations show that the hexose, fructose, permits *AtCHS* promoter activity in *At-glw* protoplasts. As with glucose, protoplasts in low concentrations of fructose show higher levels of *AtCHS* promoter activity than those in the same concentration of sucrose. This suggests that, as with glucose, this response is more sensitive to fructose than to sucrose.

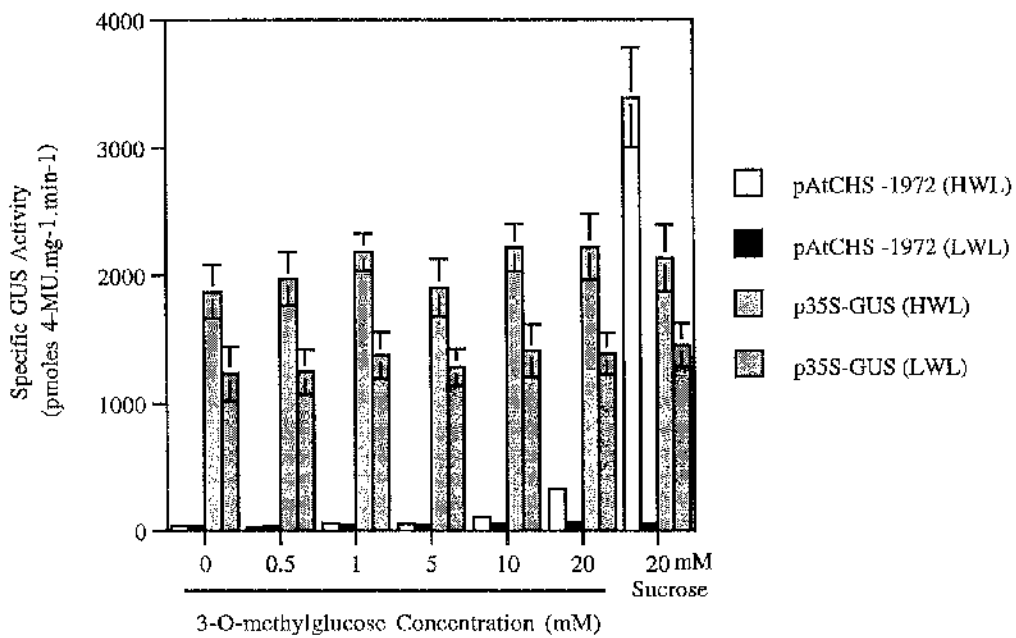
6.6 LIGHT REGULATED *AtCHS* EXPRESSION IS SIGNIFICANTLY REDUCED IN THE PRESENCE OF 3-O-METHYLGLUCOSE

In order to further analyse the sugar regulation of *AtCHS* promoter activity, experiments with sugar analogues were performed. The first of these involved performing transient expression assays with protoplasts incubated in 3-O-methylglucose. This glucose analogue is able to enter protoplasts but is thought not to be phosphorylated by hexokinase nor further metabolised. In previous experiments with photosynthetic genes and glyoxylate cycle genes, 3-O-methylglucose was unable to mediate sugar repression (Sheen, 1990; Graham *et al.*, 1994). Transient expression assays were performed as described in 6.3 substituting 3-O-methylglucose for sucrose and the results are shown in Figure 6.5.

As in previous assays, protoplasts containing the control CaMV 35S promoter: GUS reporter construct showed little change in expression levels at different concentrations of 3-O-methylglucose. Light regulation of *AtCHS* promoter activity was abolished in low concentrations of 3-O-methylglucose. In protoplasts incubated in 0.5 and 1 mM 3-O-methylglucose, in contrast to sucrose, glucose and fructose, no light induced reporter activity was observed. A slight increase in activity was observed in protoplasts under inductive light conditions incubated in 10 mM 3-O-methylglucose. Light induced

Figure 6.5 Effect of 3-O-methylglucose on light regulated *AtCHS* promoter activity in *At-glw* protoplasts

At-glw protoplasts were isolated and transfected with 20 μg of the indicated construct and transferred to HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) or LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) in medium containing a range of different concentrations of 3-O-methylglucose (0 mM to 20mM) as indicated and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



AtCHS promoter activity was observed in 20 mM 3-O-methylglucose. However, this activity was only approximately 10 % of that observed for protoplasts incubated in 20 mM sucrose. This observation may be due to 3-O-methylglucose being metabolised to some extent in *At-glw* protoplasts or perhaps the preparation of 3-O-methylglucose contained a metabolisable impurity. These results show that the *AtCHS* promoter is only light inducible under high concentrations of 3-O-methylglucose in *At-glw* protoplasts which suggests that metabolism of hexose sugars may be required for sugar regulation of *AtCHS*.

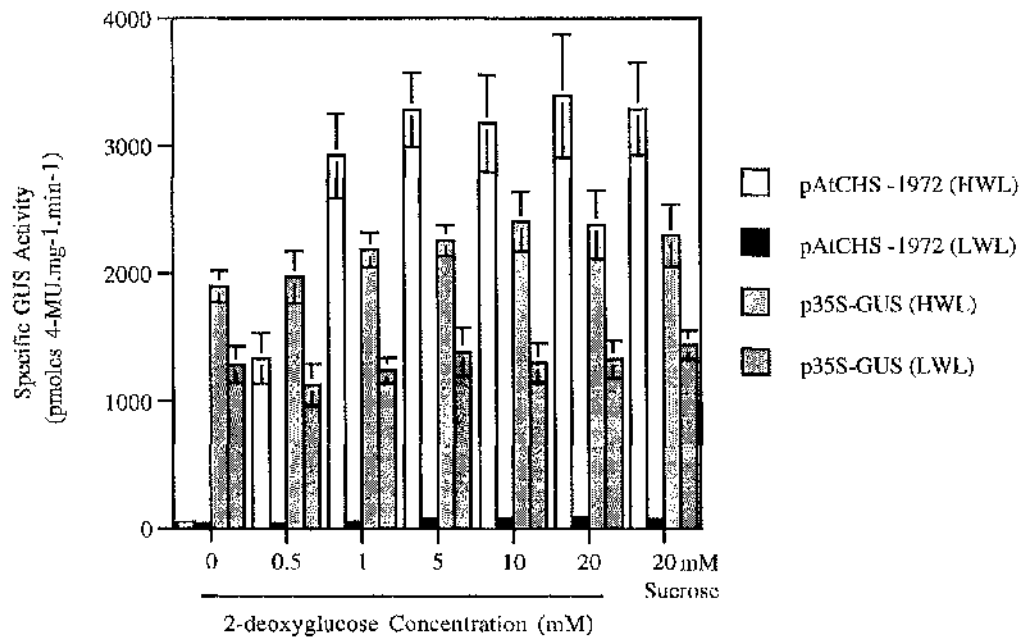
6.7 *AtCHS* EXPRESSION IS LIGHT REGULATED IN THE PRESENCE OF 2-DEOXYGLUCOSE

In order to further test the hypothesis that hexokinase was involved in sugar regulation of *AtCHS* promoter activity, transient assays with protoplasts incubated in 2-deoxyglucose were performed. 2-deoxyglucose is a glucose analogue which enters protoplasts and is thought to be phosphorylated by hexokinase but not further metabolised. In previous studies, 2-deoxyglucose was able to mediate sugar repression of gene expression (Sheen, 1990; Graham *et al.*, 1994). Transient expression assays were performed as described in 6.3 substituting 2-deoxyglucose in place of sucrose and the results are shown in Figure 6.6.

As in all previous assays, expression from the CaMV 35S promoter remained largely unaltered by changing the concentration of 2-deoxyglucose. High levels of light induced *AtCHS* expression were observed in protoplasts incubated in 1, 5, 10 and 20 mM 2-deoxyglucose. Light induced expression levels in protoplasts in 1 mM 2-deoxyglucose are approximately 90 % of those seen in protoplasts in 20 mM 2-deoxyglucose. Protoplasts in 0.5 mM 2-deoxyglucose also showed light induced

Figure 6.6 Effect of 2-deoxyglucose on light regulated *AtCHS* promoter activity in *At-glw* protoplasts

At-glw protoplasts were isolated and transfected with 20 μg of the indicated construct and transferred to HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) or LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) in medium containing a range of different concentrations of 2-deoxyglucose (0 mM to 20mM) or in 20 mM sucrose as indicated and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



expression at a level of approximately 40% of that for 20 mM 2-deoxyglucose. Light induced *AtCHS* promoter activity is higher in protoplasts in 0.5 mM 2-deoxyglucose than those in the same concentration of glucose, fructose or sucrose. These data suggest that the sensitivity of the sugar regulating mechanism to 2-deoxyglucose is greater than it is to glucose in *At-glw* protoplasts. These observations are consistent with the hypothesis that hexokinase is involved in the sugar regulation of expression from the *AtCHS* promoter. It is interesting, however, that the higher concentrations of 2-deoxyglucose do not appear to be toxic for the protoplasts. This perhaps suggests that the protoplasts may be, at least to some extent, metabolising 2-deoxyglucose.

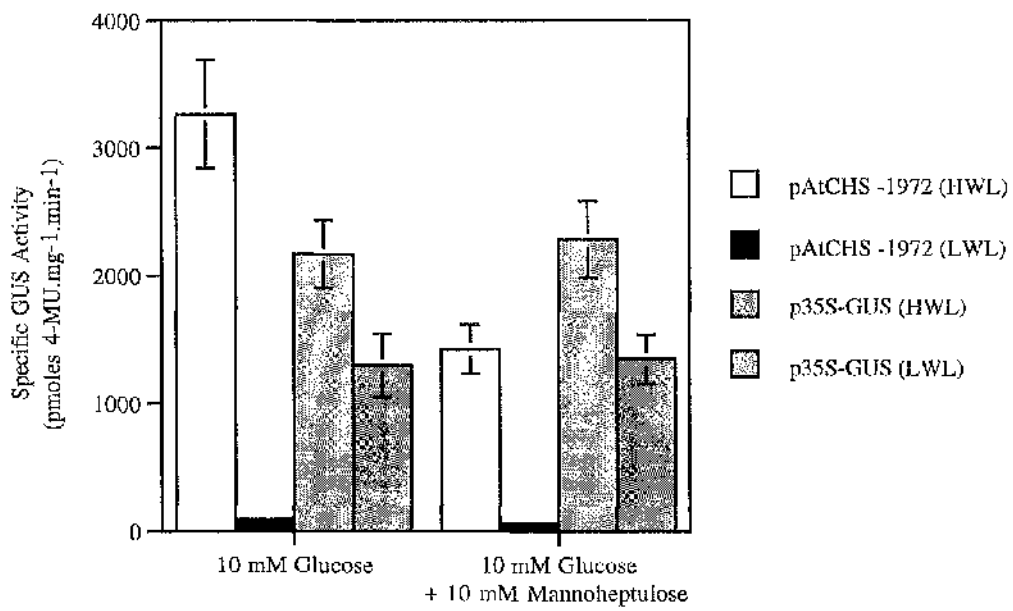
6.8 MANNOHEPTULOSE INHIBITS LIGHT REGULATED *AtCHS* EXPRESSION

Thus far, our analysis was consistent with the hypothesis that hexokinase may be involved in the sugars regulation of *AtCHS* expression. It was decided to further investigate this by using the hexokinase inhibitor mannoheptulose in transient expression studies. Mannoheptulose is a competitive inhibitor of hexokinase which was reported not to be cytotoxic to maize protoplasts in transient expression analysis (Jang and Sheen, 1994). For this analysis, *At-glw* protoplasts were transfected with either the full-length *AtCHS*: GUS construct or the control CaMV 35S: GUS reporter construct. Protoplasts were then incubated in inductive or non-inductive light conditions in medium containing either 10 mM glucose or 10 mM glucose and 10 mM mannoheptulose. The results of this analysis are shown in Figure 6.7.

In both the presence and absence of mannoheptulose, expression from the CaMV 35S promoter remains unaffected. Light induced reporter gene expression driven by the *AtCHS* promoter is present in both types of media. However, expression is significantly reduced in the presence of the hexokinase inhibitor mannoheptulose

Figure 6.7 Effect of mannoheptulose on light regulated *AtCHS* promoter activity in *At-glw* protoplasts

At-glw protoplasts were isolated and transfected with 20 μg of the indicated construct and transferred to HWL ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) or LWL ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ white light) in medium containing either 10 mM glucose or 10 mM glucose plus 10 mM mannoheptulose (as indicated) and incubated for 16 h. Protoplasts were harvested and assayed for GUS activity. The values shown are the means for six experiments. Error bars indicate the SD for each value. Where no error bars appear, the SD is too small to present accurately.



(approximately 45 % of that in the absence of mannoheptulose). This observation further supports the hypothesis that hexokinase is involved in the sugar regulation of *AtCHS* promoter activity.

6.9 DISCUSSION

Application of an homologous transient expression system has allowed us to examine the role of sugars in the regulation of *AtCHS*. These experiments indicate that protoplasts from *At-glw* cells have a requirement for sucrose with regard to the induction of *AtCHS* promoter activity. This observation is consistent with northern analysis with *Phaseolus vulgaris* protoplasts reported by Urwin and Jenkins (1997). It will be interesting to establish whether this regulation is the same in plants. The *At-glw* cell culture is routinely grown in a 3 % sucrose solution and because of this may have developed altered metabolic regulation of gene expression. However, Tsukuya *et al.* (1991) has reported the sucrose stimulation of *CHS* expression in plants.

Measurements of metabolite levels should be undertaken to confirm that the sugars and glucose analogues are being metabolised as predicted in *At-glw* protoplasts. This is essential before any serious conclusions can be drawn regarding the nature of sugar regulation of *AtCHS* promoter activity. However, although the analysis of sugar regulation of *AtCHS* expression is only just beginning, these experiments suggest that we can tentatively predict that the following observations about sugar regulation of the *AtCHS* promoter in *At-glw* protoplasts are likely to be true:

1. Light regulation of *AtCHS* promoter activity requires the presence of a carbohydrate which can be metabolised by *At-glw* protoplasts. This suggests that metabolic regulation plays a vital role in the regulation of *AtCHS*.

2. High levels of light regulation are observed in the presence of 2-deoxyglucose but not in 3-O-methylglucose and is inhibited by mannoheptulose. This is consistent with a model of hexokinase signalling being involved in the sugar regulation of *AtCHS*.

3. The minimal -164 bp *AtCHS* promoter was sufficient for sugar regulation. This observation suggests that sugar regulation is not mediated by exclusive sequence elements within the *AtCHS* promoter, but instead regulated *AtCHS* promoter activity via transcription factors which bind to common regulatory elements to control gene expression.

CHAPTER 7: FINAL DISCUSSION

7.1 INTRODUCTION

In this study, an *Arabidopsis* cell culture system was utilised to develop a light-responsive homologous protoplast transient expression system. Experiments were undertaken to optimise this system and, subsequently, transient expression assays provided a very useful approach to analyse the regions of the *AtCHS* promoter involved in UV-B and UV-A/blue light regulation. This technique was flexible enough to allow the investigation of the role of cryptochrome in this regulation through co-transfection experiments. This analysis was complemented by experiments using cryptochrome mutants. In addition, the transient expression system was used to investigate how light regulated *AtCHS* transcription is integrated with metabolic regulation. The aim of this chapter is to discuss and draw together the main conclusions of this research and summarise the prospects for future work.

7.2 A HOMOLOGOUS TRANSIENT EXPRESSION SYSTEM FOR THE FUNCTIONAL ANALYSIS OF *Arabidopsis* PROMOTERS

In this study, an efficient homologous transient expression system was developed and employed for the functional dissection of promoter elements of the *Arabidopsis CHS* gene. The isolated protoplasts responded to light with similar kinetics to both cultured cells and leaf tissue to induce *AtCHS* accumulation (Christie, 1996). In addition, the response of protoplasts to different light qualities was identical to that observed with *Arabidopsis* cells (Christie and Jenkins, 1996) and mature leaves (Fuglevand *et al.*, 1996; Jackson *et al.*, 1995; Jackson and Jenkins, 1995). That is, expression was induced by UV-B and UV-A/blue light rather than red and far-red light. The lack of response to phytochrome is expected since phytochrome only seems to control *AtCHS* gene expression during a short developmental phase after germination

(Kaiser *et al.*, 1995). Therefore, it appears that the transient expression system is appropriate for the identification of *AtCHS* promoter elements concerned with responsiveness to UV and blue light.

One advantage of a protoplast transient expression system based on cultured cells as opposed to a leaf protoplast-based system (Abel and Theologis, 1994) is that the cells from suspension culture are much more uniform. The disadvantage is that, in general, mutant protoplasts are not readily accessible. The availability of mutant protoplasts can be a valuable tool as the use of *hy4* protoplasts in this study demonstrated. However, lines of mutant cells could be initiated to overcome this disadvantage (Axelos *et al.*, 1992). The availability of an efficient and stimulus responsive transient expression system in *Arabidopsis* is valuable because of the potential to capitalise on progress in gene isolation. This is demonstrated in co-transfection "gain of function" experiments in this study where *CRY1* overexpression enhanced *AtCHS* promoter activity in response to UV-A/blue light. Furthermore, the fact that two different lines of cultured wild type *Arabidopsis* cells yielded nearly identical results gives confidence to the data obtained with the system. The flexibility of this system is clearly shown by the investigation of the role of sugars in the regulation of *AtCHS* promoter activity in this study. Clearly, the homologous transient expression system could potentially be used for the functional analysis of other *Arabidopsis* promoters with respect to different inductive stimuli. However, the given stimulus-responsive promoter would have to provide a sufficient level of induction for successful analysis in this system. Interestingly, the cultured *At-glw* cells do respond to other signals, for example low temperature (J.M. Christie, V.H. Maier and G.I. Jenkins, unpublished).

7.3 SEQUENCE ELEMENTS OF THE *AtCHS* PROMOTER INVOLVED IN LIGHT REGULATION

Sequence analysis of the *AtCHS* promoter reveals that it resembles that of *CHS* genes from several other species studied to date (Rocholl *et al.*, 1994; Schlutze-Lefert *et al.*, 1989a). In particular, the sequences of the ACE and MRE within the LRU are highly conserved (Figure 1.2) and both elements have functional characteristics similar to their parsley counterparts (Block *et al.*, 1990). It seems likely, therefore, that these elements in the *AtCHS* promoter will interact with the same classes of transcription factors that bind the LRU in parsley, that is, bZIP factors recognising the ACE, and MYB factors recognising the MRE (Feldbrügge *et al.*, 1994; Feldbrügge *et al.*, 1997). Indeed, gel shift assays indicate that *Arabidopsis* factors of the bZIP class recognise the ACE from the *AtCHS* promoter (U. Hartmann and B. Weisshaar, unpublished). *Arabidopsis* has large gene families encoding both the bZIP and MYB classes of transcription factors (Büttner and Singh, 1997; Martin and Paz-Ares, 1997; Menkens *et al.*, 1995). However, it is not known which members of these families regulate light-responsive *AtCHS* transcription. Perhaps the application of genetic approaches in *Arabidopsis* together with functional analysis in the transient expression system will resolve this question.

The transient expression system was used to define the light responsive *cis*-acting elements concerned with the transcriptional activation of *AtCHS*. To do this several chimaeric *AtCHS* promoter: GUS reporter constructs were made to dissect the regions of the promoter concerned with UV-B and UV-A/blue light regulation. These experiments demonstrate that the 48 bp LRU of the *AtCHS* gene is extremely important in the light regulation of transcription. Block mutations and gain of function experiments demonstrate that the LRU is both necessary for, and sufficient to confer, responsiveness to light in the context of the minimal -164 bp promoter. Furthermore, it mediates induction by both UV-B and UV-A/blue wavelengths and, more specifically, from the CRY1 phototransduction pathway. These results are similar to those obtained in parsley with UV-containing white light (Block *et al.*, 1990; Weisshaar *et al.*, 1991). However, the function with respect to different phototransduction pathways was not

investigated in the parsley system. It should be noted that the results presented here are somewhat different to those reported by Feinbaum *et al.* (1991). In their deletion analysis of the *AtCHS* promoter using transgenic *Arabidopsis* plants, they observed a relatively low level of blue light induction of a promoter deletion containing the LRU. It is not clear whether this reflects a difference in the responsiveness of cell culture protoplasts and *Arabidopsis* seedling tissue to light or whether there is some other explanation, for example relating to the use of particular illumination conditions and a different ecotype (*Landsberg erecta* versus Nossen).

It is clear from this study that the LRU is not the only region of the *AtCHS* promoter that can confer responsiveness to light. Mutation of both the ACE and MRE sequences, which completely inactivates LRU function in the -164 bp promoter, does not result in the inactivation of the full-length promoter and of the deletion constructs longer than -335 bp. This observation indicates that several sequences, either specifically light-responsive or quantitative, upstream of -335 may contribute to the response to light, because promoter activity increased with successively longer promoter variants containing the mutated LRU.

The deletion analysis suggested the presence of such (a) sequence element(s) between -336 and -526 of the *AtCHS* promoter. Sequence comparison at first glance indicated that this second region contains the G-box-like element located at around position -442. The fact that mutation of this sequence element did not significantly reduce the activity of the second light-responsive region showed that the sequence comparisons were misleading in this case. In contrast to the parsley *CHS* promoter which contained a second upstream LRU (outlined in Chapter 1), the upstream region of the *AtCHS* promoter does not contain a second LRU with a clearly identifiable ACE of the G-box type. This is an unexpected result given that G-box-like ACEs have been shown to be critical elements in a range plant promoters (Menkens *et al.*, 1995). Interestingly, another indication of a "non-functional" G-box-like ACE came from deletion analysis of the mustard *CHS1* promoter, but in this case no specific mutation was introduced in the G-box-like sequence (Rochoil *et al.*, 1994). Although there appears to be no specific function for the lone G-box in the *AtCHS* promoter in response to light, the possibility exists

that this element is important in the regulation of the promoter by some other stimulus. Therefore, we do not conclude that this potential ACE is necessarily non-functional.

An interesting observation is that the upstream light-regulatory region mediates qualitatively the same response to different light qualities as the LRU. Similar levels of promoter activation in response to UV-B and UV-A/blue light were observed for both the LRU and upstream regulatory region(s). Furthermore, there is no evidence to suggest that elements within the LRU mediate responses preferentially to a particular light quality. Indeed, given that no difference in responsiveness to different light qualities with any promoter fusion was observed in this study, it seems that both the UV-B and UV-A/blue light signal transduction pathways regulate the *AtCHS* promoter via transcription factors which interact with the same promoter elements. Whether exactly the same transcription factors, or different members of a family with very similar DNA-binding properties, effect the responses to both light qualities remains to be resolved. The UV-B and UV-A/blue light signal transduction pathways have been shown to be distinct, both with respect to the photoreceptors involved and specific signalling components (Christie and Jenkins, 1996; Fuglevand *et al.*, 1996). Nevertheless, it appears that the two signalling pathways converge on similar factors(s) to stimulate transcription of the *AtCHS* promoter.

7.4 UV AND BLUE LIGHT PHOTORECEPTORS INVOLVED IN *AtCHS* REGULATION

It is well established that, in *Arabidopsis*, *CHS* expression is regulated principally by UV and blue light and that phytochrome is only involved in young seedlings (Feinbaum *et al.*, 1991; Kaiser *et al.*, 1995). The CRY1 photoreceptor has been well defined functionally and shown to be involved in mediating several UV-A/blue light responses (Ahmad *et al.*, 1995; Jackson and Jenkins, 1995) and, more recently, the CRY2 photoreceptor has been cloned and partially characterised (Lin *et al.*, 1998). Experiments with *hy4* mutant seedlings, lacking detectable

levels of the CRY1 photoreceptor, showed that levels of *AtCHS* expression are reduced in blue light, undetectable in UV-A light and unaltered in UV-B light (Fuglevand *et al.*, 1996). These results suggest that with regard to *AtCHS*, CRY1 mediates part of the blue light-induced expression, all of the UV-A light-induced expression and is not involved in the UV-B light-induced expression. However, the possibility of functional redundancy between photoreceptors must be taken into consideration before any conclusions can be made regarding the role of CRY1 in the light regulation of *AtCHS* expression. The CRY1 co-transfection "gain of function" experiments in this study were designed to complement these observations and clarify the role of CRY1 in the regulation of *AtCHS* expression. These experiments, and subsequent *hy4* mutant analysis, indicate that CRY1 is the primary photoreceptor mediating the UV-A/blue light regulation of *AtCHS* transcription at the fluence rates employed. In addition, CRY1 does not appear to be involved in the regulation of *AtCHS* by UV-B light. Interestingly, the enhancement of UV-A/blue induced *AtCHS* promoter activity by CRY1 overexpression was mediated by the full-length and -164 bp promoters as well as the full-length promoter containing block mutations in the LRU. This supports the previous hypothesis that the separate phototransduction pathways regulate promoter activity via transcription factors that interact with the same sequence elements. Similar studies with CRY2 may provide a valuable insight into the role, if any, this photoreceptor plays in controlling the regulation of *AtCHS*.

The availability of *fha1* mutants allowed us to investigate the role of the CRY2 photoreceptor in the UV and blue light regulation of *AtCHS* in this study. However, northern analysis indicated that, at least at the fluence rates tested, the CRY2 photoreceptor does not appear to be involved in the regulation of *AtCHS* by UV-A/blue or UV-B light. Initially, it seemed likely that functional redundancy between photoreceptors was perhaps masking the effect of the *fha1* mutation. However, similar analysis with *fha1/hy4* provided no further evidence of function in *AtCHS* regulation. Indeed, low levels of UV-A/blue light induced *AtCHS* transcription were detected in these double mutant seedlings suggesting that even in the absence of CRY1, the *fha1* mutation had no detectable effect on *AtCHS* expression. The recent report that CRY2 is degraded in blue light at moderate and high fluence rates suggests that CRY2 is unlikely to be

involved in the regulation of *AtCHS* (Lin *et al.*, 1998). This data appears to suggest that the CRY2 photoreceptor functions only at very low fluence rates, for example in hypocotyl elongation, and/or is capable of functioning at very low concentrations at higher fluence rates.

The roles of the CRY1 and CRY2 photoreceptors with regard to the regulation of *AtCHS* expression by the synergistic interaction between the UV and blue light signalling pathways was investigated using the cryptochrome mutants. UV-A and blue light each show a separate synergistic response with UV-B light to induce *AtCHS* expression (Fuglevand *et al.*, 1996). These synergistic responses are distinct and additive and in this study we investigated the effect of *hy4* and *pha1* mutations on these combined synergisms. These experiments show that neither the *hy4* nor the *pha1* mutations had any effect on the UV-A/blue and UV-B synergistic induction of *AtCHS* expression under the light conditions tested. Interestingly, the *pha1/hy4* double mutant seedlings also showed an unaltered response to simultaneous UV-A/blue and UV-B light. This suggests that photoreceptor(s) other than CRY1 and CRY2 is/are mediating the synergistic interactions of UV-A and blue light with UV-B.

Fuglevand *et al.* (1996) found that the UV-A signal for the synergism is a transient one because a synergistic interaction between UV-A and UV-B was only observed when the two treatments were given simultaneously. In contrast, the blue signal is a stable one and a blue light treatment before the UV-B treatment still induced a synergistic response. Fuglevand *et al.* (1996) showed that neither synergistic response is altered in *hy4* mutants and the results of this study are in agreement with that observation. However, an investigation into the effect of the double cryptochrome mutation on the separate UV-A/UV-B and blue/UV-B synergisms could perhaps reveal whether functional redundancy is masking the effects of a single mutation on either of the synergistic responses.

7.5 SUGAR REGULATION OF *AtCHS* PROMOTER ACTIVITY

In this study, the metabolic regulation of *AtCHS* was analysed using the transient expression system. These experiments indicated that protoplasts from At-glw cells have a requirement for sucrose with regard to the induction of *AtCHS* expression. This observation was consistent with northern analysis reported by Urwin and Jenkins (1997). Although the aim of this study was primarily to investigate the light regulation of *AtCHS* expression, it is important not to overlook the role of metabolic regulation since *in vivo* this will be amongst the most important factors in *AtCHS* regulation. Furthermore, it serves to demonstrate how *AtCHS* regulation is likely to be controlled by a wide variety of stimuli *in vivo*. In addition, it demonstrates the flexibility of the transient expression system to facilitate the investigation of different signals controlling expression from a given promoter.

Interestingly, in this study the minimal *AtCHS* promoter was sufficient to mediate the sucrose requirement for promoter activity. This suggests, as previously discussed with different light qualities, that this signal is transduced via transcription factor(s) binding to the sequence elements within the LRU. This indicates that the sugar regulation signal does not mediate control through exclusive sequence elements. This leads us to consider whether this is true for all of the signals that regulate *AtCHS* expression. If this is the case, the degree of complexity at the level of the signal transduction pathways involved is fascinating.

It is, perhaps, worth questioning whether the regulation of promoter activity observed in this study reflects the situation in intact plants. Indeed, part of the reason for investigating the nature of the sugar regulation of *AtCHS* transcription was to examine whether the high levels of apparently light-induced promoter activity were in some way also being stimulated by the high concentrations of sucrose in which protoplasts were incubated. It appears more likely that there is a sucrose requirement for *AtCHS* induction given that no differences in promoter activity were observed in low fluence rate light conditions between samples in different concentrations of sucrose. Furthermore, light-induced promoter activity drops very sharply in concentrations

of sucrose below 1 mM, and above 1 mM promoter activity remains relatively similar in increasing concentrations of sucrose. This sharp drop seems more consistent with requirement for sucrose than a stimulation by sucrose. The fact that the pattern of *AtCHS* expression in these experiments is consistent with that reported for protoplasts from mature leaf tissue by Urwin and Jenkins (1997), suggests that promoter regulation in the transient expression system, as with regulation by different light qualities, accurately reflects that in intact plants.

Furthermore, analysis using glucose analogues in this study suggests a model of sugar regulation consistent with hexokinase signalling. However, the use of glucose analogues to investigate the nature of the sugar regulation of *AtCHS* has its shortcomings. Measurements of metabolite levels in protoplasts are essential in order to confirm that the analogues used are being metabolised as expected. Such measurements are required before any firm conclusions can be drawn regarding the nature of the sugar regulation of *AtCHS* promoter activity.

7.6 FUTURE WORK

Although this study has provided important information regarding UV and blue light regulation of transcription in *Arabidopsis*, further experiments are required to extend this work and to examine other aspects of the control of *AtCHS* regulation. In order to extend this work, continued use of the transient expression system to perform "gain of function" experiments to further analyse the role of CRY2 in *AtCHS* regulation would be useful. More detailed analysis into the separate UV-A/UV-B and blue/UV-B synergism responses using the cryptochrome mutants may provide valuable insights into the roles of the photoreceptors involved in these responses. The UV-B and UV-A/blue signal transduction pathways controlling *AtCHS* regulation are under detailed investigation and have been shown to be distinct, both with respect to the photoreceptors involved and specific signalling components (Christie and Jenkins, 1996; Funglevand *et al.*, 1996). This study suggests that the two signalling pathways converge on similar factor(s), members of the bZIP and MYB families of transcription factors, to stimulate

transcription of the *AtCHS* promoter. There is evidence that one or more factors are synthesised *de novo* after illumination, because *AtCHS* activation by UV-B and UV-A/blue light in *Arabidopsis* is inhibited by cycloheximide (Christie and Jenkins, 1996). It would be interesting, now, to identify the effector proteins, in particular the transcription factors, at which the signalling pathways appear to converge.

The identification of negative regulators of *AtCHS* transcription will also provide useful insights into the control of *AtCHS* gene expression in *Arabidopsis*. For example, the *icx1* mutant identifies a negative regulator of *AtCHS* expression, which shows increased responsiveness to light (Jackson *et al.*, 1995). Since the *icx1* mutant shows elevated *AtCHS* gene expression in both UV-B and UV-A/blue light (H.K. Wade and G.I. Jenkins, unpublished), it seems likely that ICX1 functions in both of these light signalling pathways. The identification and characterisation of further mutants defining regulators of *AtCHS* expression is an important direction of future research.

The isolation of genes encoding additional photoreceptors of UV-B, UV-A and blue light and the identification and characterisation of corresponding mutants will be essential to advance our understanding in this area. The availability of a variety of phytochrome mutants has greatly enhanced understanding of phytochrome function. The analysis of the functions of such gene products could clearly be performed in the homologous transient expression system described here and thus allow the definition of the molecular targets of the light signalling pathways.

In a broader context, the transient expression system also provides a suitable means to study other signal-mediated responses, such as *AtCHS* induction by low temperature (Christie, 1996). Different promoters could be analysed provided levels of induction are sufficiently high and protoplasts provide an accurate reflection of regulation in mature *Arabidopsis* plants, as in the case of light and sugar regulation of *AtCHS*. The inherent flexibility of the transient expression system make it suitable for studying a wide variety of signal-mediated responses.

REFERENCES

- Abel, S., and Theologis, A. (1994) *Plant J.* **5**, 421-427.
- Adamse, P., Jaspers, P.A.P.M., Kendrick., R.E., and Koorneef, M. (1987) *J. Plant Physiol.* **127**, 481-491.
- Adamse, P., Kendrick., R.E., and Koorneef, M. (1988) *Phytochem. Phytobiol.* **48**, 833-841.
- Ahmad, M., and Cashmore, A.R. (1993) *Nature* **366**, 162-166.
- Ahmad, M., Lin, C., and Cashmore, A.R. (1995) *Plant J.* **8**, 653-658.
- Ahmad, M., and Cashmore, A.R. (1996) *Plant Mol. Biol.* **30**, 851-861.
- Ahmad, M., and Cashmore, A.R. (1997) *Plant Journal* **11**, No.3, 421-427
- Ahmad, M., Jarillo, J.A., Smirnova, O., Cashmore, A.R. (1998a) *Molecular Cell* **1**, No.7, 939-948
- Ahmad, M., Jarillo, J.A., and Cashmore, A.R. (1998b) *Plant Cell* **10**, 197-207
- Algarra, P., Linder, S., and Thümmler, F. (1993) *FEBS Lett.* **315**, 69-73.
- Allen, G., J., Muir, S.R., and Sanders, D. (1995) *Science* **268**, 735-737.

- Amodeo, G., Srivastava, A., and Zeiger, E. (1992) *Plant Physiol.* **100**, 1567-1570.
- Ang, L-H., and Deng, X-W. (1994) *Plant Cell* **6**, 613-628.
- Assman, S.M., Simoncini, L., and Schroeder, J.I. (1985) *Nature* **318**, 285-287.
- Assman, S.M., and Schwartz, A. (1992) *Plant Physiol.* **98**, 1349-1355.
- Aukerman, M.J., Hirschfeld, M., Wester, L., Weaver, M., Clack, T., Amasino, R.M., Sharrock, R.A. (1997) *Plant Cell* **9**, No.8, 1317-1326
- Axelos, M., Curie, C., Mazzolini, L., Bardet, C., and Lescure, B. (1992) *Plant Physiol. Biochem.* **30**, 123-128.
- Ballare, C.L., Scopel, A.L., Roush, M.L., Radosevich, S.R. (1995) *Plant Physiol.* **93**, 584-592.
- Batschauer, A., Ehmann, B., and Schäfer, E. (1991) *Plant Mol. Biol.* **16**, 175-185.
- Batschauer, A. (1993) *Plant J.* **4**, 705-709.
- Batschauer, A., Rocholl, M., Kaiser, T., Nagatani, A., Furuya, M., and Schäfer, E. (1996) *Plant J.* **9**, 63-69.
- Barnes, S.A., Quaggio, R.B., and Chua, N-H. (1995) *Phil. Trans. R. Soc. Lond. B* **350**, 67-74.

- Block, A., Dangl, J.L., Hahlbrock, K., and Schultz-Lefert, P. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5387-5391.
- Blum, W., Hinsch, K-D., Schultz, G., and Weiler, E.W. (1988) *Biochem. Biophys. Res. Comm.* **156**, 954-959.
- Bolwell, G.P. (1995) *TIBS* **20**, 492-495.
- Bossen, M.E., Kendrick, R.E., and Vredenberg, W.J. (1990) *Physiol. Plant.* **80**, 55-62.
- Bowler, C., Neuhaus, G., Yamagata, H., and Chua, N-H. (1994a) *Cell* **77**, 73-81.
- Bowler, C., Yamagata, H., Neuhaus, G., and Chua, N-H. (1994b) *Genes Dev.* **8**, 2188-2202.
- Briggs, W.R., and Iino, M. (1983) *Phil. Trans. R. Soc. B* **303**, 347-359.
- Bush, D.S. (1993) *Plant Physiol.* **103**, 7-13.
- Cabrera y Poch, H.L., Peto, C.A., and Chory, J. (1993) *Plant J.* **4**, 671-682.
- Cashmore, A.R. (1997) *Plant Cell And Environment* **20**, No.6, pp.764-767
- Castle, L.A., and Meinke, D.W. (1994) *Plant Cell* **6**, 25-41.
- Chappell, J., and Hahlbrock, K. (1984) *Nature* **311**, 76-78.

Chamovitz, D.A., Wei, N., Osterlund, M.T., von Arnim, A.G., Staub, J.M., Matsui, M., Deng, X-W. (1996) *Cell* **86**, 115-121

Chattopadhyay, S., Ang, L.H., Puente, P., Deng, X.W., Wei, N. (1998) *Plant Cell* **10**, No.5, 673-683.

Chaudhury, A.M., Letham, S., Craig, S., Dennis, E.S. (1993) *Plant Journal* **4**, 907-916.

Cherry, J.R., Hondred, D., Walker, J.M., Keller, K.M., Hershey, H.P., and Vierstra, R.D. (1993) *Plant Cell* **5**, 565-575.

Cho, M.H., and Spalding, E.P. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 8134-8138.

Chory, J., Peto, C., Ashbaugh, M., Saganich, R., Pratt, L., and Ausubel, F. (1989a) *Plant Cell* **1**, 867-880.

Chory, J., Peto, C., Feinbaum, R., Pratt, L., and Ausubel, F. (1989b) *Cell* **58**, 991-999.

Chory, J., and Peto, C.A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 8776-8780.

Chory, J. (1992) *Development* **115**, 337-354.

Chory, J. (1993) *TIG* **9**, 167-172.

Chory, J., Nagpal, P., Peto, C.A. (1991) *Plant Cell* **3**, No.5, 445-459

- Christie, J.M., Jenkins, G.I. (1996) *Plant Cell* **8**, No.9, 1555-1567.
- Christie, J.M. (1996) Ph.D. Thesis, University of Glasgow.
- Church, G.M., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1991-1995.
- Clack, T., Matthews, S., and Sharrock, R.A. (1994) *Plant Mol. Biol.* **25**, 413-427.
- Conley, T.R., and Shih, M-C. (1995) *Plant Physiol.* **108**, 1013-1022.
- Coupland, G. (1995) *TIG* **11**, 393-
- Damm, B., Schmidt, R., and Willmitzer, L. (1989) *Mol. Gen. Genet.* **217**, 6-12.
- Dangl, J.L., Hauffe, K.D., Lipphardt, S., Hahlbrock, K., and Scheel, D. (1987) *EMBO J.* **6**, 2551-2556.
- Dangl, J.L., Hahlbrock, K., and Schell, J. (1989) In *Plant Nuclear Genes and their Expression* Vol. 6, ed. I.K. Vasil and J. Schell., pp.155-173. New York, Academic Press.
- Dehesh, K., Franci, C., Parks, B.M., Seeley, K.A., Short, T.W., Tepperman, J.M., and Quail, P.H. (1993) *Plant Cell* **5**, 1081-1188.
- Deng, X-W., Matsui, M., Wei, N., Wagner, D., Chu, A.M., Feldmann, F.A., and Quail, P.H. (1992) *Cell* **71**, 791-801.

- Deng, X-W. (1994) *Cell* **76**, 423-426.
- Deng, X.W., Caspar, T., Quail, P.H. (1991) *Genes & Development* **5**, No.7, 1172-1182.
- Desnos, T., Orbovic, V., Bellini, C., Kronenberger, J., Caboche, M., Traas, J., Hofte, H. (1996) *Development* **122**, No.2, 683-693
- Dietrich, A., Meyer, J.E., and Hahlbrock, K. (1990) *J. Biol. Chem.* **265**, 6360-6368.
- Dharmawardhane, S., Rubinstein, B., and Stern, A.I. (1989) *Plant Physiol.* **89**, 1345-1350.
- Diekman J., and Hammer, P.E. (1995) *Plant Physiol.* **108**, 47-57.
- Drøbak, B.J. (1992) *Biochem. J.* **288**, 679-712.
- Droge-Laser, W., Kaiser, A., Lindsay, W., Halkier, B.A., Loake, G.J., Doerner, P., Dixon, R.A., Lamb, C. (1997) *EMBO J.* **16**, No.4, 726-738
- Doelling, J.H., and Pikaard, C.S. (1993) *Plant Cell Rep.* **12**, 241-244.
- Edgerton, M.D., and Jones, A.M. (1992) *Plant Cell* **4**, 161-171.
- Edgerton, M.D., and Jones, A.M. (1993) *Biochemistry* **32**, 8239-8243.
- Ensminger, P.A., and Schäfer, E. (1992) *Phytochem. Phytobiol.* **55**, 437-447.

- Faktor, O., Loake, G., Dixon, R.A., and Lamb, C.J. (1997) *Plant J.* **11**, 1105-1113.
- Feinbaum, R.L., and Ausubel, F.M. (1988) *Mol. Cell Biol.* **8**, 1985-1992.
- Feinbaum, R.L., Storz, G., and Ausubel, F.M. (1991) *Mol. Gen. Genet.* **226**, 449-456.
- Feldbrugge, M., Sprenger, M., Dinkelbach, M., Yazaki, K., Harter, K., and Weisshaar, B. (1994) *Plant Cell* **6**, 1607-1621.
- Feldbrugge, M., Sprenger, M., Hahlbrock, K., and Weisshaar, B. (1997) *Plant J.* **11**, 1079-1093.
- Feldbrugge, M., Hahlbrock, K., Weisshaar, B. (1996) *Mol. & Gen. Genet.*, **251**, 619-627
- Foster, R., Izawa, T., and Chua, N-H. (1994) *FASEB J.* **8**, 192-200.
- Frohmeier, H., Ehmann, B., Kretsch, T., Rocholl, M., Harter, K., Nagatani, A., Furuya, M., Batschauer, A., Hahlbrock, K., and Schäfer, E. (1992) *Plant J.* **2**, 899-906.
- Frohmeier, H., Hahlbrock, K., and Schäfer, E. (1994) *Plant J.* **5**, 437-449.
- Fromm, H., Katagiri, F., Chua, N.H. (1991) *Mol. & Gen. Genet* **229**, 181-188
- Fuglevand, G., Jackson, J.A., and Jenkins, G.I. (1996) *Plant Cell* **8**, 2347-2357.

- Furuya, M. (1993) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 617-645.
- Gabrielsen, O.S., Sentenac, A., Fromageot, P. (1991) *Science* **253**, 1140-1143
- Galland, P., and Lipson, E.D. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 104-108.
- Galland, P., and Senger, H. (1988a) *J. Photochem. Photobiol. B* **1**, 277-294.
- Galland, P., and Senger, H. (1988b) *Photochem. Photobiol.* **48**, 811-820.
- Gallagher, S., Short, T.W., Ray, P.M., Pratt, L.H., and Briggs, W.R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8003-8007.
- Gao, J., and Kaufman, L.S. (1994) *Plant Physiol.* **104**, 1251-1257.
- Gautier, H., Vavasseur, A., Lasceve, G., and Boudet, A. (1992) *Plant Physiol.* **98**, 34-38.
- Gepstein, S., Jacobs, M., and Taize, L. (1982) *Plant Sci. Lett.* **28**, 63-72.
- Goff, S.A., Klein, T.M., Roth, B.A., Fromm, M.E., Cone, K.C., Radicella, J.P., Chandler, V.L. (1990) *EMBO J.* **9**, 2517-2522
- Goff, S.A., Cone, K.C., Chandler, V.L. (1992) *Genes & Dev.* **6**, 864-875
- Goff, S.A., Cone, K.C., Fromm, M.E. (1991) *Genes & Dev.* **5**, 298-309

- Graham, I.A. (1996) *Res. in Microbiol.* **147**, 572-580.
- Graham, I.A., Denby, K.J., and Leaver, C.J. (1994) *Plant Cell* **6**, 761-772.
- Green, R., and Fluhr, R. (1995) *Plant Cell* **7**, 203-212.
- Grierson, C., Du, J-S., Zabala, M., Beggs, K., Smith, C., Holdsworth, M., and Bevan, M. (1994) *Plant J.* **5**, 815-826.
- Guo, A., Durner, J., Klessig, D.F. (1998) *Plant Journal* **15**, No.5, 647-656.
- Hagar, A., Brich, M., and Bazlen, I. (1993) *Planta* **190**, 120-126.
- Hagar, A.F , and Spitzer, J.A. (1992) *Cell Calcium* **13**, No.2, 123-130.
- Hanks, S.K., and Hunter, T. (1995) *Faseb Journal* **9**, No.8, 576-596.
- Hahlbrock, K., and Scheel, D. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 347-369.
- Hahlbrock, K., Scheel, D., Logemann, E., Nürnberger, T., Parniske, M., Reinfold, S., Sacks, W.R., and Schmelzner, E. (1995)) *Proc. Natl. Acad. Sci. USA* **92**, 4150-4157.
- Harter, K., Talke-Messer, C., Barz, W., and Schäfer, E. (1993) *Plant J.* **4**, 507-516.

- Harter, K., Kircher, S., Frohnmeyer, H., Krenz, M., Nagy, F., and Schäfer, E. (1994) *Plant Cell* **6**, 545-559.
- Hou, Y., von Arnim, A.G., and Deng, X-W. (1993) *Plant Cell* **5**, 329-339.
- Howe, K.M., Reakes, C.F.L., Watson, R.J. (1990) *EMBO J.* **9**, 161-169
- Huala, R., Liscum, M., and Briggs, W.R. (1997) *Science* **278**, 2120-2123
- Huang, N., Chandler, J., Thomas, B.R., Koizumi, N., and Rodriguez, R.L. (1993) *Plant Mol. Biol.* **23**, 737-747.
- Hughes, D.A. (1994) *Nature* **369**, 187-188.
- Hunter, T., and Karin, M. (1992) *Cell* **70**, 375-387.
- Iino, M. (1990) *Plant Cell Environ.* **13**, 633-650.
- Ishida, S., Takahashi, Y., and Nagata, T. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1152-1156.
- Ishiguro, S., and Nakamura, K. (1992) *Plant Mol. Biol.* **18**, 97-108.
- Jackson, J.A., Fuglevand, G., Brown, B.A., Shaw, M.A., and Jenkins, G.I. (1995) *Plant J.* **8**, 369-380.
- Jackson, J.A., and Jenkins, G.I. (1995) *Planta* **197**, 233-239.

Jacobs, M., Thelen, M.P., Farndale, R.W., Astle, M.C., and Rubery, P.H. (1988) *Biochem. Biophys. Res. Comm.* **155**, 1478-1484.

Jang, J.-C., and Sheen, J. (1994) *Plant Cell* **6**, 1665-1679.

Jenkins, G.I. (1991) In *Developmental Regulation of Plant Gene Expression*, ed. D. Grierson, pp. 1-41. Blackie, Glasgow.

Jenkins, G.I., Christie, J.M., Fuglevand, G., Long, J.C., and Jackson, J.A. (1995) *Plant Sci.* **112**, 117-138.

Jenkins, G.I. (1997) *Plant Cell Envir.* **20**, 773-778.

Jorns, M.S., Wang, B., Jordan, S.P., and Chanderkar, L.P. (1990) *Biochemistry* **29**, 552-561.

Kaiser, T., and Batschauer, A. (1995) *Plant Mol. Biol.* **28**, 231-243.

Kaiser, T., Emmeler, K., Kretsch, T., Weisshaar, B., Schäfer, E., and Batschauer, A. (1995) *Plant Mol. Biol.* **28**, 219-229.

Kaufman, L.S. (1993) *Plant Physiol.* **102**, 333-337.

Kaufman, L.S. (1994) *Phytochem. Phytobiol. B: Biol.* **22**, 3-7.

Khare, M., and Gurusparad, K.N. (1993) *Plant Sci.* **91**, 1-5.

Khurana, J.P., and Poff, K.L. (1989) *Planta* **178**, 400-406.

Khurana, J.P., Ren, Z., Steinitz, B., Parks, B., Best, T.R., and Poff, K.L. (1989) *Plant Physiol* **91**, 685-689.

Kingston, R.E., Bunker, C.A., Imbalzano, A.N. (1996) *Genes & Development* **10**, 905-920

Klimezak, L.J., Schindler, U., and Cashmore, A.R. (1992) *Plant Cell* **4**, 87-98.

Koes, R.E., Quattrocchio, F., and Mol, J.N.M. (1993) *BioEssays* **16**, 123-132.

Konjevic, R., Steinitz, B., and Poff, K.L. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9876-9880.

Konjevic, R., Khurana, J.P., and Poff, K.L. (1992) *Pytochem. Phytobiol.* **55**, 789-792.

Koorneef, M., Rolff, E., and Spruit, C.J.P. (1980) *Z. Pflanzenphysiol.* **100**, 147-160.

Koorneef, M. (1981) *Arabidopsis Information Service* **18**, 45-51.

Koorneef, M., Hanhart, C.J., Vanderveen, J.H. (1991) *Molecular & General Genetics* **229**, No.1, 57-66.

Kreuzaler, F., Ragg, H., Fautz, E., Kuhn, D.N., Hahlbrock, K. (1983) *P.N.A.S.* **80**, 2591-2593

- Kubasek, W.I., Shirley, B.W., McKillop, A., Goodnan, H.M., Biggs, W.R., and Ausubel, F.M. (1992) *Plant Cell* **4**, 1229-1236.
- Kwok, S.F., Piekos, B., Misera, S., Deng, X-W. (1996) *Plant Physiol* **110**, No.3, 731-742
- Lagarias, J.C., and Lagarias, D.M. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5778-5780.
- Lesnick, M.L., and Chandler, V.L. (1998) *Plant Physiol.* **117**, 437-445
- Li, H-M., Altschmied, L., and Chory, J. (1994) *Genes Dev.* **8**, 339-349.
- Li, Y.F., Sancar, A. (1990) *Biochemistry* **29**, No.24, 5698-5706.
- Lin, C., Ahmad, M., Gordon, D., and Cashmore, A.R. (1995a) *Proc. Natl. Acad. Sci. USA* **92**, 8423-8427.
- Lin, C., Robertson, D.E., Ahmad, M., Raibekas, R.A., Jorns, S., Dutton, L., and Cashmore, A.R. (1995b) *Science* **269**, 968-970.
- Lin, C.T., Yang, H.Y., Guo, H.W., Mockler, T., Chen, J., Cashmore, A.R. (1998) *P.N.A.S* **95**, No.5, 2686-2690.
- Lin, C.T., Ahmad, M., Cashmore, A.R. (1996) *Plant Journal* **10**, No.5, 893-902.
- Lipphardt, S., Brettschneider, R., Kreuzaler, F., Schell, J., and Dangl, J.L. (1988) *EMBO J.* **7**, 4027-4033.

Liscum, E., and Hangarter, R.P. (1991) *Plant Cell* **3**, 685-694.

Liscum, E., and Hangarter, R.P. (1993) *Planta* **191**, 214-221.

Liscum, E., and Hangarter, R.P. (1994) *Plant Cell Environ.* **17**, 639-649.

Liscum, E., and Briggs, W.R. (1995) *Plant Cell* **7** 473-485.

Lois, R. (1994) *Planta* **194**, 498-503.

Lois, R., and Buchanan, B.B. (1994) *Planta* **194**, 504-509.

Lorenzi, R., Ceccarelli, N., Lercari, B., Gualtieri, P. (1994) *Phytochemistry* **36**, No.3, 599-600.

Liu, X.J., Prat, S., Willmitzer, L., and Frommer, W.J.(1990) *Mol. Gen. Genet.* **223**, 401-406.

Ma, H., Yanofsky, M.F., and Meyerowitz, E.M. (1990)) *Proc. Natl. Acad. Sci. USA* **87**, 3821-3825.

Ma, H., Yanofsky, M.F., and Huang, H. (1991) *Gene* **107**, 189-195.

Maas, C., Schaal, S., and Werr, W. (1990) *EMBO J.* **9**, 3447-3452.

Malhotra, K., Kim, S-T., Batschauer, A., Dawut, L., and Sancar, A. (1995) *Biochemistry* **34**, 6892-6899.

- Mancinelli, A.L. (1994) In *Photomorphogenesis in Plants*, ed. R.E.Kendrick and G.H.M. Kronenburg, pp.211-269. Kluwer Academic Publishers, Dordrecht.
- Martin, C., and Paz-Ares, J. (1997) *TIG* **13**, 67-73.
- Matsui, M., Stoop, C.D., von Arnim, A.G., Wei, N., and Deng, X-W. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 4239-4243.
- May, M.J., and Leaver, C.J. (1993) *Plant Physiol.* **103**, 621-627.
- McNellis, T.W., von Arnim, A.G., Araki, T., Komeda, Y., Misera S., Deng, X-W. (1994a) *Plant Cell* **6**, 487-500.
- McNellis, T.W., von Arnim, A.G., Deng, X-W. (1994b) *Plant Cell* **6**, 1391-1400.
- McNellis, T.W., Torii, K.U., Deng, X-W. (1996) *Plant Cell* **8**, 1491-1503.
- McCormac, A.C., Wagner, D., Boylan, M.T., Quail, P.H., Smith, H., and Whitelam, G.C. (1993) *Plant J.* **4**, 19-27.
- McCurdy, D.W., and Pratt, L.H. (1986) *Planta* **167**, 330-336.
- Menkens, A.E., Schindler, U., and Cashmore, A.R. (1995) *TIBS* **20**, 506-510.
- Meyerowitz, E.M. (1989) *Cell* **56**, 263-269.
- Millar, A.J., McGrath, R.B., and Chua, N-H. (1994) *Mol. Gen. Genet.* **28**, 325-349.

Miséra, S., Müller, A.J., Weiland-Heidecker, U., And Jürgens, G. (1994) *Mol. Gen. Genet* **224**, 242-252.

Mol, J, Jenkins, G.L., Schafer, E., Weiss, D. (1996) *CRIT. REVS. PLANT SCI.* **15**, No.5-6, 525-557

Morse, M.J., Crain, R.C., and Satter, R.L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7075-7078.

Morse, M.J., Crain, R.C., Coté, G.G., and Satter, R.L. (1989) *Plant Physiol.* **89**, 724-727.

Murphy, T.M., and Huerta, A.J. (1990) *Physiol. Plant.* **78**, 247-253.

Nagano, Y., Murai, N., Matsuno, R., and Sasaki, Y. (1993) *Plant Cell Physiol.* **34**, 447-455.

Nagano, Y., Okada, Y., Narita, H., Asaka, Y., and Sasaki, Y. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 6314-6318.

Nagatani, A., and Sakamoto, K. (1996) Light-dependent nuclear localisation of Phytochrome B. *The Japan-United States Bi-national Workshop*: Tsukuba, Ibaraki 305, Japan. [Abstract]

Neuhaus, G., Bowler, C., Kern, R., and Chua, N-H. (1993) *Cell* **73**, 937-952.

Nishizaki, Y. (1988) *Plant Cell Physiol.* **29**, 1041-1046.

- Nishizaki, Y. (1994) *Plant Cell Physiol* **35**, 841-844.
- Ohl, S., Hahlbrock, K., and Schäfer, E. (1989) *Planta* **177**, 228-236.
- Ohl, S., Hedrick, S.A., Chory, J., and Lamb, C.J. (1990) *Plant Cell* **2**, 837-848.
- Oyama, T., Shimura, Y., Okada, K. (1997) *Genes & Development* **11**, No.22, 2983-2995
- Palmer, J.M., Short, T.W., and Briggs, W.R. (1993) *Plant Physiol.* **102**, 1219-1225.
- Pang, Q., and Hays, J.B. (1991) *Plant Physiol.* **95**, 536-543.
- Parks, B.M., and Quail, P.H. (1991) *Plant Cell* **3**, 1177-1186
- Parks, B.M., and Quail, P.H. (1993) *Plant Cell* **5**, 39-48.
- Pepper, A., Delaney, T., Washburn, T., Poole, D., and Chorey, J. (1994) *Cell* **78**, 109-116.
- Peters, J.L., van Tuinen, A., Adamse, P., Kendrick, R.E., and Koorneef, M. (1989) *J. Plant Physiol.* **134**, 661-666.
- Polisensky, D.H., and Braam, J. (1996) *Plant Physiol.* **111**, 1271-1279.

Putterill, J., Robson, F., Lee, K., Simon, R., and Coupland, G. (1995) *Cell* **80**, 847.

Quail, P.H. (1991) *Annu. Rev. Genet.* **25**, 389-409.

Quail, P.H. (1994) In *Photomorphogenesis in Plants*, ed. R.E.Kendrick and G.H.M. Kronenberg, pp.211-269. Kluwer Academic Publishers, Dordrecht.

Quail, P.H., Boylan, M.T., Parks, B.M., Short, T.W., Xu, Y., and Wagner, D. (1995) *Science* **268**, 675-680.

Quiñones, M.A., and Zeiger, E. (1994) *Science* **264**, 558-560.

Raghavendra, A.S. (1990) *Plant Cell Environ.* **13**, 105-110.

Raz, V., and Fluhr, R. (1993) *Plant Cell* **5**, 523-530.

Reed, J.W., Nagpal, P., Poole, D.S., Furuya, M., and Chory, J. (1993) *Plant Cell* **5**, 147-157.

Reed, J.W., Nagatani, A., Elich, J.D., Fagan, M., and Chory, J. (1994) *Plant Physiol.* **104**, 1139-1149.

Reymond, P., Short, T.W., and Briggs, W.R. (1992a) *Plant Physiol.* **100**, 655-661.

Reymond, P., Short, T.W., Briggs, W.R., and Poff, K.L. (1992b) *Proc. Natl. Acad. Sci. USA* **89**, 4718-4721.

Rocholl, M., Talke-Messerer, C., Kaiser, T., and Batschauer, A. (1994) *Plant Sci.* **97**, 189-198.

Romero, L.C., Sommer, D., Gotor, C., and Song, P-S. (1991) *FEBS Lett.* **282**, 431-436.

Romero, L.C., and Lam, E. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1465-1469.

Roux, S.J., Wayne, R.O., and Datta, N. (1986) *Physiol. Plant.* **66**, 344-348.

Russ, U., Grolig, F., Wagner, G. (1991) *Planta* **184**, No.1, 105-112.

Saikumar, P., Murali, R., Reddy, E.P. (1990) *P.N.A.S.* **87**, 8452-8456.

Sainz, M.B., Grotewold, E., Chandler, V.L. (1997) *Plant Cell* **9**, 611-625

Salomon, M., Zacherl, M., Rudiger, W. (1996) *Planta* **199**, No.3, 336-342.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Cold Spring Harbour, New York. Cold Spring Harbour Laboratory Press.

Sancar, A (1994) *Biochemistry* **33**, 2-9.

Schmelzer, E., Jahnen, W., and Hahlbrock, K. (1988) *Proc. Natl. Acad. Sci. USA* **88**, 2989-2993.

- Schmidt, W., Galland, P., Senger, H., and Furuya, M. (1990) *Planta* **182**, 375-381.
- Schneider-Poetsch, H.A.W. (1992) *Photochem. Photobiol.* **56**, 839-846.
- Schroeder, J.I., Schmidt, C., and Sheaffer, J. (1993) *Plant Cell* **5**, 1831-1841.
- Schulze-Lefert, P., Becker-André, M., Schulz, W., Hahlbrock, K., and Dangle, J.L. (1989a) *Plant Cell* **1**, 707-714.
- Schulze-Lefert, P., Dangle, J.L., Becker-André, M., Hahlbrock, K., and Schulz, W. (1989b) *EMBO J.* **8**, 651-656.
- Schwartz, A., Ilan, N., and Assmann, S.M. (1991) *Planta* **183**, 590-596.
- Senger, H., and Schmidt, W. (1994) In *Photomorphogenesis in Plants*, ed. R.E.Kendrick and G.H.M. Kronenburg, pp.211-269. Kluwer Academic Publishers, Dordrecht.
- Shacklock, P.S., Read, N.D., and Trewavas, A.J. (1992) *Nature* **358**, 753-755.
- Sharma, Y.K., and Davis K.R. (1994) *Plant Physiol.* **105**, 1089-1096.
- Sharrock, R.A., and Quail, P.H. (1989) *Genes and Dev.* **3**, 1745-1757.
- Sheen, J. (1990) *Plant Cell* **2**, 1027-1038.
- Sheen, J. (1993) *EMBO J.* **12**, 3497-3505.

- Sheen, J. (1994) *Photosynthesis Res.* **39**, 427-438.
- Shimada, H., Iba, K., and Takamiya, K-I. (1992) *Plant Cell Physiol.* **33**, 471-475.
- Shimazaki, K., Iino, M., and Zeiger, E. (1986) *Nature* **319**, 324-326.
- Shimazaki, K-I., Kinoshita, T., and Nishimura, M. (1992) *Plant Physiol.* **99**, 1461-1421.
- Shimazaki, K-I., Omasa, K., Kinoshita, T., and Nishimura, M. (1993) *Plant Cell Physiol.* **34**, 1321-1327.
- Shinkle, J.R., and Jones, L.R. (1988) *Plant Physiol.* **86**, 960-966.
- Short, T.W., and Briggs, W.R. (1990) *Plant Physiol.* **92**, 179-185.
- Short, T.W., Porst, M., and Briggs, W.R. (1992) *Photochem. Photobiol.* **55**, 773-781.
- Short, T.W., Reymond, P., and Briggs, W.R. (1993) *Plant Physiol.* **101**, 647-655.
- Short, T.W., and Briggs, W.R. (1994) *Annu. Rev. Plant Physiol. Mol. Biol.* **45**, 143-171.
- Simon, R., Igeno, M.I., and Coupland, G. (1996) *Nature* **384**, 59-63
- Smith, H. (1997) *Plant Cell And Environment* **20**, No.6, pp.788-793

Solano, R., Nicto, C., Avila, J., Canas, L., Diaz, I., Pazares, J. (1995) *EMBO J.* **14**, 1773-1784

Somerville, C.R., Somerville, S.C., and Orgen, W.L. (1981) *Plant Sci. Lett.* **21**, 89-96.

Sommers, D., and Song, P.S. (1994) *Protein Express. Purif.* **5**, 402-408.

Spalding, E.P., and Cosgrove, D.J. (1989) *Planta* **178**, 407-410.

Spalding, E.P., and Cosgrove, D.J. (1992) *Planta* **188**, 199-205.

Spanswick, R.M. (1981) *Annu. Rev. Plant Physiol.* **32**, 267-289.

Srivastava, and Zeiger, (1995) *Planta* **196**, 445-449.

Stapleton, A.E. (1992) *Plant Cell* **4**, 1353-1358.

Stapleton, A.E., Walbot, V. (1994) *Plant Physiol* **105**, No.3, 881-889.

Staub, J.M., and Deng, X-W (1996) *Plant Cell* **8**, 698-704.

Steinitz, B., Ren, Z., and Poff, K.L. (1985) *Plant Physiol.* **77**, 248-251.

Suzuki, K., Fukuda, Y., and Shishi, H. (1995) *Plant Cell Physiol.* **36**, 281-289.

Szekeres, M., Nemeth, K., Koncz, Z., Mathur, J., Kauschmann, A., Altmann, T., Redei, G.P., Nagy, F., Schell, J., Koncz, C. (1996) *Cell* **85**, 171-182.

- Takahashi, Y., Sakai, T., Ishida, S., Nagata, T. (1995) *P.N.A.S* **92**, 6359-6363
- Takeda, S., Mano, S., Ohto, M-H., and Nakamura, K. (1994a) *Plant Physiol.* **106**, 567-574.
- Takeda, J., Obi, I., and Yoshida, K. (1994b) *Physiol. Plant.* **91**,517-521.
- Tamari, G., Borochoy, A., Atzorn, R., and Weiss, D. (1995) *Physiol. Plant.* **94**, 45-50.
- Tester, M. (1990) *New Phytol.* **114**, 305-340.
- Terryn, N., Van Montague, M., and Inzé, D. (1993) *Plant Mol. Biol.* **22**, 143-152.
- Terzaghi, W.B., and Cashmore, A.R. (1995a) *Annu. Rev. Plant Physiol. Mol. Biol.* **46**, 445-474.
- Terzaghi, W.B., and Cashmore, A.R. (1995b) *Cur. Biol.* **5**, 466-468.
- Tevini, M., and Teramura, A.H. (1989) *Phytochem. Phytobiol.* **50**, 479-487.
- Thompson, W.F., and White, M.J. (1991) *Annu. Rev. Plant Physiol. Mol. Biol.* **42**, 423-466.
- Thummler, F., Dufner, M., Kreisel, P., and Dittrich, P. (1992) *Plant Mol. Biol.* **20**, 1003-1017.

Thummler, F., Algarra, P., and Fobo, G.M. (1995) *FEBS Lett.* **357**, 149-155.

Torres, J.T., Block, A., Hahlbrock, K., and Somssich, I.E. (1993) *Plant J.* **4**, 587-592.

Trewavas, A., and Gilroy, S. (1991) *TIG* **7**, 356-361.

Trezzini, G.F., Horrichs, A., and Somssich, I.E. (1993) *Plant Mol. Biol.* **21**, 385-389.

Tsukaya, H., Ohshima, T., Naito, S., Chino, M., and Komeda, Y. (1991) *Plant Physiol.* **97**, 1414-1421.

Van Tunen, A.J., Hartman, S.A., Mur, L.A., Mol, J.N.M. (1989) *Plant Mol. Biol.* **12**, 539-551

Vani, T., and Raghavendra, A.S. (1989) *Plant Physiol.* **90**, 59-62.

Vani, T., and Raghavendra, A.S. (1992) *J. Exp. Bot.* **43**, 291-297.

Vera-Estrella, R., Barkla, B.J., Higgins, V.J., and Blumwald, E. (1994) *Plant Physiol.* **104**, 209-215.

von Arnim, A.G., and Deng, X-W. (1994) *Cell* **79**, 1035-1045.

von Arnim, A.G., and Deng, X-W. (1993) *Journal Of Biol. Chem.* **268**, 19626-19631

Wada, M., and Sugai, M. (1994) Photobiology of Ferns. In *Photomorphogenesis in Plants*, ed. R.E.Kendrick and G.H.M. Kronenburg, pp.211-269. Kluwer Academic Publishers, Dordrecht

Wada, M., Kanegae, T., Nozue, K., and Fukuda, S. (1996) The Gene Families of Phytochrome and Blue-Light-Absorbing Pigments in *Adiantum capillus-veneris*. In *New Prospects of Photobiology Conference Proceedings*, Okazaki (Abstract). NIBB, Japan.

Wagner, D., and Quail, P.H. (1995)) *Proc. Natl. Acad. Sci. USA* **92**, 8596-8600.

Wanner, L.A., Li, G., Ware, G.L., Somssich, I.E., and Davis, K.R. (1995) *Plant Mol. Biol.* **27**, 327-338.

Warpeha, K.M.F., Hamm, H.E., Rasenick, M.M., and Kaufman, L.S. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8925-8929.

Warpeha, K.M.F., Kaufman, L.S., and Briggs, W.R. (1992) *Phytochem. Photobiol.* **55**, 595-603.

Wei, N., Chamovitz, D.A., and Deng, X-W. (1994a) *Cell* **78**, 117-124.

Wei, N., Kwok, S.F., von Arnim, A.G., Lee, A., McNellis, T.W., Piekos, B., and Deng, X-W. (1994b) *Plant Cell* **6**, 629-643.

Weiss, C.A., Garnaat, C.W., Mukai, K., and Ma, H. (1994)) *Proc. Natl. Acad. Sci. USA* **91**, 9554-9558.

Weisshaar, B., Armstrong, G.A., Block, A., da Costa e Silva, O., and Hahlbrock, K. (1991a) *EMBO J.* **10**, 1777-1786.

Weisshaar, B., Block, A., Armstrong, G.A., Hermann, A., Schulze-Lefert, P., and Hahlbrock, K. (1991b) In *Molecular Biology of Plant Development, Symp. Soc. Exp. Biol.* XLV, ed. G.I. Jenkins and W. Schuch, pp. 191-210. Cambridge: Company of Biologists.

Weisshaar, B., and Jenkins, G.I. (1998) *Curr. Opinion Plant Biol.* **1**, 251-257

Whitelam, G.C., and Harberd, N.P. (1994) *Plant Cell And Environment* **17**, No.5, 615-625.

Whitelam, G.C., Johnson, E., Peng, J.R., Carol, P., Anderson, M.L., Cowl, J.S., Harberd, N.P. (1993) *Plant Cell* **5**, No.7, 757-768.

Xu, Y., Parks, B.M., Short, T.W., and Quail, P.H. (1995) *Plant Cell* **7**, 1433-1443.

Yoshida, K., Nagano, Y., Murai, N., and Sasaki, Y. (1993)) *Proc. Natl. Acad. Sci. USA* **90**, 6636-6640.

Yoshioka, H., Shiraishi, T., Kawamata, S., Nasu, K., Yamada, T., Ichinose, Y., and Hachiro, O. (1992) *Plant Cell Physiol.* **33**, 201-204.

