Regulation of chalcone synthase gene expression in wild-type and mutant *Arabidopsis*

by

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Abbreviations

ACE	ACGT-containing element
ADH	Alcohol dehydrogenase
Amp	Ampicillin, an antibiotic
bZIP	basic leucine zipper
CAB	chlorophyll a/b binding protein
CD	Cytosine deaminase
CFI	Chalcone isomerase, previously called CHI
CHS	Chalcone synthase
CK2	casein kinase II
CodA	Cytosine deaminase coding sequence
cop	*constitutive photomorphogenesis* mutants
CPRF	common plant regulatory factor
CRY	Cryptochrome
det	*de-etiolated* mutants
DFR	Dihydroflavonol reductase
DNA	deoxyribonucleic acid
DPI	diphenylene iodonium
E. coli	*Escherichia coli*
EMS	ethyl methanesulphonate
5-FC	5-fluorocytosine
5-FU	5-fluorouracil
fhy1	putative phytochrome A signal transduction mutant
fus	*fusca* mutants
GFP	green fluorescent protein
gl	*glabra* mutants
Gn	Gentamycin, an antibiotic
GUS	β–glucuronidase
hy3	*long hypocotyl 3* mutant, also known as *phyB*
hy4	*long hypocotyl 4* mutant, also known as *cry1*
hy5	*long hypocotyl 5* mutant
HIR	high irradience response
icx1	*increased chalcone synthase expression* mutant
Kan	Kanamycin, an antibiotic
LF	low fluence
LRU	Light Responsive Unit

LTP	lipid transfer protein
MRE	MYB recognition element
NM4	non- mutant line containing *CHS-GUS*
nph	*non-phototropic hypocotyl* mutants
NPPB	5-nitro-2-(3-phenylpropylamino)-benzoic acid
PAL	L-phenylalanine ammonia-lyase
Pfr	far red light absorbing form of phytochrome
phyA	phytochrome A mutant
phyB	phytochrome B mutant
Pr	red light absorbing form of phytochrome
rbcS	small unit of rubisco
RNA	Ribonucleic Acid
ttg	*transparent testa, glabra* mutant
tt	*transparent testa* mutants
TUB	α-tubulin
UV-A	Ultraviolet A light, wavelength 320-390 nm
UV-B	Ultraviolet B light, wavelength 280-320 nm
VLFR	very low fluence response
WT	wild type

Abstract

The regulation of chalcone synthase (*CHS*) gene expression in *Arabidopsis thaliana* plants was investigated. *icx1* (*increased chalcone synthase expression*, Jackson *et al.*,1995, *Plant J.* 8; 369-380), a mutant altered in the light regulation of *CHS* expression, was studied in detail. Mature *icx1* leaf tissue was found to have increased *CHS* expression compared to that of wild type plants in UV-A/blue, UV-A and UV-B light. Dark grown *icx1* seedlings displayed an increase in *CHS* expression in the above light qualities and also in far red light. These results indicate that ICX1 acts as a negative regulator downstream of phytochrome, cryptochrome and UV-B photoreceptors and in both mature leaves and seedlings. The synergistic effect of UV-B plus blue light or UV-B plus UV-A light on *CHS* expression is not altered in the *icx1* mutant. A model is presented to explain the position of ICX1 in relation to the inductive and synergistic pathways. Genetic analysis shows that *hy5* is epistatic to *icx1* and it is proposed that the HY5 transcription factor functions downstream of ICX1 in the light signalling pathway. In addition, the cold induction of *CHS* is increased in *icx1*. Therefore ICX1 also regulates a non-light signalling pathway. *icx1* has increased expression of other genes involved in flavonoid biosynthesis, encoding dihydroflavonol reductase and chalcone isomerase. The ICX1 gene product does not affect the light regulation of *CAB* and *rbcS* genes which are expressed in mesophyll cells. It is proposed that ICX1 acts principally in the epidermis.

The induction of *CHS* expression by UV light was found to be regulated by phytochrome. When mutant plants lacking phytochrome B or both phytochrome B and phytochrome A, were exposed to UV-A light, the resultant *CHS* expression was lower than that seen in the wild type plants.A red light exposure given to wild type plants prior to UV-A illumination resulted in an increase in *CHS* expression. In the *phyA phyB* double mutant the overall induction of *CHS* was reduced but the increase in *CHS* expression in red

pretreated plants was retained. This increase is most likely regulated by something other than phytochrome A or B. In UV-B light the opposite effect was seen, with an increase in *CHS* expression in the absence of phytochrome B and in the absence of both phytochromes A and B.

The basis for a mutant screen to isolate positive regulators of *CHS* expression is presented. Transgenic plants containing a cytosine deaminase gene fused to a *CHS* promoter were produced. These can now be screened for mutants with low *CHS* expression in response to particular stimuli.

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1.1 Introduction

Light is central to many aspects of plant growth and development and investigation of the means of relaying information about the light environment is vital to aid the understanding of plant responses. Germinating seedlings need to detect the onset of light to trigger a change from skotomorphogenesis (etiolated growth) to photomorphogenic growth and the concomitant changes involved in developing photosynthetic competence. During its life, a plant is constantly responding to alterations in the light environment and such aspects as stem elongation, leaf positioning and flowering are all affected. The presence of neighbouring or overshadowing plants also affects the light environment, with the growth strategy altering in response to the signals received.

Plants also recognise and respond to various other environmental signals, such as nutrient availability, pathogen attack and wounding. Light, however, plays a significant role in a the life cycle of a plant and as such there are a number of ways a plant detects light (Kendrick and Kronenberg, 1994). A given plant must be able to discriminate between the different light qualities and respond appropriately depending on the developmental stage of the plant at which the light is detected.

The light quality a plant is normally exposed to during its natural life cycle tends to vary, depending on a large number of external factors. Some of these factors include the time of the day, weather patterns and shading by other plants. Longer term effects also affect light quality. The amount of UV-B light reaching the earth is increasing due to depletion of the stratospheric ozone layer (Landry *et al.*, 1995). This increased level of UV-B light is significant as UV-B light can cause damage to plant DNA (Ballaré *et al.*, 1996) and increased UV-B must be dealt with either by better repair mechanisms and/or better prevention of the damage to DNA. Damage prevention could, for example, be by absorption of the UV-B rays at the epidermis, limiting the amount of

penetrating UV light and thus limiting the damage at the DNA level. Absorption of UV-B can be mediated by flavonoids (Li *et al.*, 1993; Lois, 1994) and by sinapic acid esters (Landry *et al.*, 1995).

Plants are able to distinguish light qualities and quantities as they possess an array of photoreceptors which are sensitive to specific wavelengths of light. The wavelength designations of light qualities used in this study are:- UV-B, 280-320 nm; UV-A, 320-390 nm; blue, 390-500 nm; red, 600-700 nm; and far red, 700-800 nm.

There are thought to be several classes of photoreceptor present in plants: the mainly red and far red light absorbing phytochromes; the blue and UV-A photoreceptors, including cryptochromes and a photoreceptor for phototropism; and the, as yet unidentified, UV-B photoreceptor(s).

1.2 Photoreceptors

The ability of plants to detect and respond to light was investigated by Darwin (1880). It was noted that plants displaying heliotropism (phototropism) were incredibly sensitive to light. "A pot with seedlings of *Phalaris Canariensis*, which had been raised in darkness, was placed in a completely darkened room, at 12 feet from a small lamp. After 3 h. the cotyledons were doubtfully curved towards the light, and after 7 h. 40 m. from the first exposure, they were all plainly, though slightly, curved towards the lamp...the light was so obscure that we could not see the seedlings themselves...but could just distinguish a line made [on white paper] with Indian ink."

The phytochromes were the first family of photoreceptors to be isolated (Butler *et al.*, 1959). There is a family of five of these red/far red absorbing receptors in *Arabidopsis* (Bowler and Chua, 1994) and phytochrome families exist in many other plant species. In *Arabidopsis* blue light photoreceptors have been isolated, including two cryptochromes and a putative blue light photoreceptor for phototropism. Cryptochrome 1 (CRY1) was first identified by Ahmad *et al.* (1995). A second cryptochrome (CRY2) was isolated in 1996 due to

its sequence similarity to *CRY1* (Lin *et al.*, 1996). Cryptochromes have since been found in other plants and also in humans and mice. Also absorbing blue light, the *Arabidopsis* NPH1 protein is thought to be a photoreceptor for phototropism, involved in detecting a blue light signal and mediating the signal transduction leading to the subsequent hypocotyl curvature toward that light source (Liscum and Briggs, 1995). Zeaxanthin has been implicated in the blue light regulation of stomatal opening (Zeiger and Zhu, 1998). The third class of photoreceptor that is thought to exist is that of the as yet unidentified UV-B photoreceptor(s) (Christie and Jenkins, 1996; Jenkins *et al.*, 1997).

1.2.1 Phytochromes

Red and far-red light have long been known to have biological activity in plants, Smith (1975) cites the work of Flint and McAllister as the initiation of study on photomorphogenesis. Their work in 1935-1937 indicated a role for red light in enhancing germination of lettuce seedlings and for far red light in inhibiting the germination. Red and far red light affect germination, cell elongation, chlorophyll accumulation and flowering time amongst a number of other responses (Chory *et al.*, 1996).

Phytochrome exists in two different forms, a red absorbing form (Pr) and a far red light absorbing form (Pfr). On absorption of red light by Pr it is converted into the Pfr form (Smith, 1975). Under natural light conditions there is not a complete conversion of all the phytochrome from one light absorbing form to the other. Even in red light and far red light the phytochrome content is not 100% converted to the Pfr or Pr form. Casal (1995) calculated that the red light sources they used produced 87% Pfr, that the far red light source produced 2.7% Pfr and that blue light resulted in 43% Pfr.

A phytochrome photoreceptor molecule is made up of a dimer of chromoproteins. Each chromoprotein is made up of a linear tetrapyrrole chromophore covalently liked to a polypeptide of about 125 kDa. Phytochromes contain two major structural domains:- a globular NH_2-terminal

where the chromophore is located, which is the photosensory domain of the phytochrome; and the COOH-terminal which is more extended, contains a putative histidine kinase sequence and is involved in dimerisation (Quail, 1997).

PHYA is found in large amounts in etiolated seedlings, it is activated by far red light illumination, irreversibly triggering physiological and molecular responses (Furuya and Schäfer, 1996). PHYA is a Type I phytochrome, classed as such because it is light labile, unlike the phytochromes B to E which are classed as Type II, light stable, phytochromes. PHYA is thought to undergo a conformational change when activated, and that this active form is degraded by a ubiquitin-based system (Clough and Vierstra, 1997). PHYB is active in the Pfr form, after exposure to red light. It is possible to return PHYB to the inactive, Pr state, by exposure to far red light. However, some very low fluence responses (VLFR) of red light result in the same ratio of Pfr which is obtained by a far red light exposure and therefore is not reversible. Similarly high irradiance responses (HIR) requiring prolonged exposure to either red or far red light are not reversible due to the response requirement for continuous light (reviewed in Batschauer, 1998; Batschauer, 1999; Chory *et al.*, 1996; Quail, 1997).

In *Arabidopsis* there is a gene family of five phytochromes, named A, B, C, D and E (Clack *et al.*, 1994), which absorb mainly in and mediate responses to the red/far red region of the spectrum, though phytochromes absorb in the blue and UV region of the spectrum. The different phytochromes have overlapping roles in *Arabidopsis* though they are expressed at varying levels in the *Arabidopsis* plant.

The availability of phytochrome null mutants is helping to identify the roles for the individual phytochromes in various responses. Such null mutant research and also studies investigating downstream components of the phytochrome signalling pathways, have been able to indicate a much greater role of PHYA in red light mediated responses than had been initially thought. A recent study has reported the ability of rice PHYA to partially compliment

the *phyB* phenotype in *Arabidopsis* (Halliday *et al.*, 1999). Also, investigation of *spa* mutants, which are thought to act early in PHYA signal transduction, showed that PHYA is involved in a number of VLFR in continuous red light (Hoecker *et al.*, 1998). Barnes *et al.* (1996b) were also investigating a mutant which acted downstream from PHYA, the *fhy1* mutant. Their study indicated that an early induction of *chalcone synthase* (*CHS*) gene expression by red light seen in 4 day old etiolated seedlings after 2 hours of illumination, was almost entirely mediated by PHYA, but that the *fhy1* mutant was not affected in blue light induction of *CHS*. The *fhy1* mutant acts at a branch point in the PHYA downstream signalling pathway. PHYA regulated gene expression is not all affected by the *fhy1* mutation. Although red light induction of *CHS* expression is altered in the mutant, as is expression of the next step in the flavonoid biosynthetic pathway, *chalcone flavone isomerase* (*CFI*), and the rubisco small subunit (*rbcS*), the induction of chlorophyll a/b binding (*CAB*) transcripts is no different from that of the wild type plants.

In PHYB mediated signalling pathways a gene, *RED1*, has been implicated in red light responses (Wagner *et al.*, 1997). PHYB overexpressing lines were mutated and screened for plants which were revertants of the PHYB overexpression phenotype. It was suggested that RED1 may be an early signalling component of PHYB mediated pathways.

Recently, phytochromes A and B have been found to interact with PIF3, a protein which is found to be constitutively localised in the nucleus (Ni *et al.*, 1998). It is thought that this signalling component may be involved in the phytochrome regulation of the circadian clock. The light dependent movement of phytochrome into the nucleus from the cytoplasm would allow for light dependent interaction of phytochrome with PIF3. The evidence for the nuclear localisation of PHYB in light is increasing. A nuclear localisation signal was found on the C-terminal region of PHYB (Sakamoto and Nagatani, 1996). Following this, utilisation of a fusion protein between PHYB and the green fluorescent protein (GFP) enabled observation of PHYB-GFP translocation into

the nucleus in response to both white and red light (Yamaguchi *et al.*, 1999).

One possible mode of action of phytochromes is that they function as dimers. If this is the case and PHYA can function as a heterodimer, while PHYB can only function as a homodimer an explanation of the different levels of sensitivity of the two may be provided (Furuya and Schäfer, 1996). PHYA has sensitivity four orders of magnitude greater than that of PHYB.

Phytochromes are emerging as possible protein kinases. The discovery of a cyanobacterial phytochrome which displays light activated histidine kinase activity (Yeh *et al.*, 1997) has indicated an evolutionary relationship to phytochrome signalling in plants. In plants however, kinase activity of photoreceptors has not been demonstrated (reviewed in Elich and Chory, 1997; Quail, 1997).

1.2.2 Blue/UV-A Photoreceptors

In plants, a number of responses are mediated by UV/blue light, including: regulation of hypocotyl extension growth; phototropism; regulation of stomatal opening; and anthocyanin accumulation.

1.2.2.1 Cryptochromes

The first blue light receptor isolated, cryptochrome 1, (CRY1) was detected via mutant studies. The *hy4* mutant was isolated due to its long hypocotyl phenotype in blue light (Ahmad and Cashmore, 1993; Koornneef *et al.*, 1980). The amino acid sequence of the wild type gene altered in the mutant was elucidated and compared to other known sequences. Ahmad and Cashmore found that the 681 amino acid protein showed very high homology over the first 500 amino acids with microbial photolyases which catalyse blue light mediated repair of DNA damage. The area of greatest homology (up to 80%) was with regions of known to be involved in chromophore binding (Ahmad and Cashmore, 1993). Further work by Lin *et al.* (1995b) showed that CRY1, the protein encoded by the *HY4* gene, did not seem to posses any photolyase activity. The absorption properties of CRY1 are dependent on the

bound chromophore flavin adenine dinucleotide (FAD), and its stability as flavosemiquinone (FADH•). The absorption properties of FADH• allow for the range of wavelengths in which responses are mediated by CRY1, which could not be explained otherwise by FAD binding alone. Malhotra *et al.* (1995) suggest that a pterin is also probably bound as a chromophore. Point mutations induced in the CRY1 protein have shown that mutations throughout the various domains affect function of the protein (Ahmad *et al.*, 1995).

The CRY1 protein is involved in the accumulation of anthocyanins in young seedlings (Ahmad *et al.*, 1995) and *hy4* mutants flower much later than wild type plants as a result of the loss of blue light promotion of flowering (Bagnall *et al.*, 1996). In addition there are alterations in extension growth responses other than that of hypocotyl extension (Jackson and Jenkins, 1995).

A second cryptochrome (CRY2) was detected via sequence similarity to the *CRY1* gene (Lin *et al.*, 1996). CRY2 retains sequence similarity to the N-terminal (chromophore binding) region of CRY1, but the C-terminal region of CRY2 is shorter than, and shows less homology to, that of CRY1. CRY2 also binds at least a flavin as a chromophore (Lin *et al.*, 1996).

As in the phytochromes, there seems to be some overlap of function between the family members. CRY2 is able to mediate some of the same responses as CRY1. One of the differences between the two cryptochromes is that CRY2 is light labile, with levels of CRY2 protein decreasing after exposure to activating wavelengths of light (green, blue and UV-A) (Lin *et al.*, 1998). CRY2 accumulates in dark grown seedlings and may play a similar role to that of PHYA, in response to low fluence rates of light (Ahmad *et al.*, 1998a; Lin *et al.*, 1998). Like CRY1, CRY2 is also involved in regulating flowering time, indeed, *cry2* was found to be allelic to the late flowering *fha* mutant (Guo *et al.*, 1998). Investigation of blue light induced anthocyanin accumulation and hypocotyl elongation in the *cry1cry2* double mutant indicates an overlapping role of both cryptochromes. The double mutant displayed a decreased level of anthocyanin accumulation and longer hypocotyls than the *cry1* parent when

grown in blue light (Ahmad *et al.*, 1998c).

Cryptochrome-like genes have been isolated in a number of other plant species, including members of the mono- and dicotyledons, ferns and algae. To investigate the role of cryptochrome in other plant species, the C-terminal portion of the tomato *CRY1* gene, *TCRY1*, was expressed in the antisense direction in tomato (Ninu *et al.*, 1999). This study indicated that *TCRY1* is involved in anthocyanin accumulation, but not in the regulation of carotenoid or chlorophyll levels. The second positive phototropic curvature was also unaffected.

Cryptochromes are not restricted to plant species, human and mouse homologues have been isolated and appear to play a role in regulation of circadian rhythmicity (Thresher *et al.*, 1998). That cryptochromes affect circadian responses in mice via mCry2 is also mirrored in *Arabidopsis*. *cry1* and *cry2* mutants both show altered circadian responses, as CRY1 acts to transmit both high and low fluence blue light signals to the circadian clock. CRY2 is thought to have an effect on photoperiodic timing through a gating effect by the circadian clock on the CRY2 mediated signal (Somers *et al.*, 1998).

1.2.2.2 Phototropism Photoreceptor

Phototropism, the tendency of a plant to grow towards light, is mediated by green, blue and UV-A light. In *cry1* and *cry1cry2* mutants the phototropic response remains (Christie *et al.*, 1998; Liscum and Briggs, 1996). Four non-phototropic hypocotyl (*nph*) mutants have been isolated which are altered in the phototropic response (Liscum and Briggs, 1996). The *nph1* mutant lacks a phosphoprotein which is phosphorylated upon blue light irradiation. The NPH1 protein is a serine/threonine kinase and contains regions with sequence similarity (61%) to LOV (light, oxygen, voltage) motifs, sensitive to redox altering environmental factors (Huala *et al.*, 1997).

The other mutants altered in phototropism, *nph2*, *nph3* and *nph4* act downstream from this phosphoprotein as the phosphoprotein is found to be

intact in these mutants. *nph4* is additionally altered in gravitropism, and the protein product may be acting at a later stage of the signal transduction, possibly directly at the point of establishing differential growth (Liscum and Briggs, 1996).

Some confusion arose after a study by Ahmad *et al.* (1998c) reported that mutants lacking both the CRY1 and CRY2 photoreceptors did not show any first-positive phototropic curvature in the double mutants. In single mutants lacking either CRY1 or CRY2, the phototropic response is retained, which suggested that either cryptochrome is sufficient for phototropic responses. It was concluded that the removal of both cryptochromes prevents the phosphorylation of the NPH1 protein, which was then suggested to be the next step in the phototropic signal cascade. The lack of phototropic response in the double mutant, along with the observation of the presence of CRY1 in membrane preparations containing the phosphoprotein, led Ahmad *et al.* to suggest that the phosphorylation of NPH1 upon exposure to blue light in membrane fractions was a result of phosphorylation by either CRY1 or CRY2. This conclusion implied that NPH1 was not a photoreceptor, but the next downstream component in the phototropism pathway. However, further studies by Christie *et al.* (1998) involving the expression of NPH1 protein in an insect system, imply that NPH1 does have the properties of a photoreceptor and that it does not require cryptochrome for phosphorylation (Christie *et al.*, 1998). A further investigation of blue light mediated phototropism in 2 and 3 day old seedlings indicate that *cry1cry2* double mutants displayed wild type levels of curvature, and that NPH1 is phosphorylated in the *cry1cry2* double mutant (Lascève *et al.*, 1999).

The recombinant NPH1 isolated from the insect cells was found to autophosphorylate in blue light. It was noted that NPH1 had a non covalently bound flavin mononucleotide associated with it which could act as a light harvesting chromophore. In addition, the fluorescence excitation spectrum of the protein extracted from the NPH1 expressing insect cells was similar to that

of the action spectra for phototropism.

1.2.2.3 Zeaxanthin- Stomatal Blue Light Chromophore?

The *Arabidopsis npq1* mutant fails to display blue light induced stomatal opening (Zeiger and Zhu, 1998). *npq1* is defective in violaxanthin de-epoxidase (Niyogi *et al.*, 1998), the first enzyme involved in the formation of zeaxanthin. The cryptochrome and the phototropism mutants were not altered in their stomatal opening in response to blue light, as measured by CO_2 flux and leaf stomatal conductance (Lascève *et al.*, 1999). This supports the idea that a separate blue light photoreceptor is involved in stomatal opening in *Arabidopsis* plants.

1.2.3 UV-B Photoreceptor(s)

There is evidence for the presence of at least one UV-B photoreceptor, mediating responses to the spectrum range of 280-320 nm (Jenkins *et al.*, 1997). So far there has been no direct evidence for the existence of a UV-B photoreceptor, but the circumstantial evidence for the existence is quite strong. In tomato, physiological experiments indicate that hypocotyl elongation is inhibited at a maximum of 300 nm by a specific UV-B photoreceptor (Ballaré *et al.*, 1995). It has also been suggested that photolyase is induced by UV-B (Pang and Hays, 1991).

UV-B irradiation of plants has been shown to reduce photosynthetic capacity of pea seedlings, as well as acting to stimulate the expression of *CHS*. That alteration of gene expression might be regulated by exposure to UV-B through carbohydrate changes in the plant rather than by some other mechanism has been investigated in pea. Mackerness *et al.* (1997) report that the UV-B induced decrease in expression of nuclear encoded photosynthetic genes is not mediated through changes in the amount of carbohydrate present in the leaves, so the possibility of a UV-B photoreceptor remains.

The cryptochromes do not absorb in the UV-B region of the spectrum, the *hy4* mutants, lacking cryptochrome 1, still show induction of *CHS*

transcripts by UV-B light, equivalent to that of wild type induction (Fuglevand *et al.*, 1996). Further work by Valentine (1998) has indicated that in both the *cry2* single mutant and in the *cry1cry2* double mutants, the *CHS* expression in response to UV-B illumination is unaltered compared to wild type. The UV-B mediated increase in *CHS* expression is therefore not effected by either cryptochrome 1 or 2.

Phytochromes are known to absorb in the UV-B range and a recent paper by Kim *et al.* (1998) has shown that UV-B responses at low fluence (LF) rates (0.01-1.0 μmol m^{-2} s^{-1}) produce photomorphogenic changes in wild type *Arabidopsis* seedlings. These photomorphogenic alterations, including hypocotyl growth inhibition, are not seen in the *phyA phyB* double mutant, which argues for the involvement of either one or both of these phytochromes in the LF response of seedlings to UV-B. It does not, however, rule out the existence of a UV-B photoreceptor which either interacts with phytochromes or mediates UV-B responses to higher fluence levels.

1.3 Interaction of Photoreceptor Signals

Signals downstream from the various photoreceptors are known to interact with one another, both within a photoreceptor family (Casal, 1996) and with other photoreceptor families (Ahmad and Cashmore, 1997; Fuglevand *et al.*, 1996; Liscum and Briggs, 1996). Phytochrome has recently been shown to interact with cryptochrome altering the resulting response produced (Ahmad *et al.*, 1998b).

After germination, the first stage of light detection by a plant involves multiple photoreceptors. When a seed germinates in the dark, it follows a developmental pathway known as skotomorphogenesis. In skotomorphogenesis (an appropriate response for seeds germinating under soil) the seedling remains etiolated, the hypocotyl extends, the seedling retains the apical hook and does not undergo any photosynthetic cellular differentiation. When the cotyledons emerge from the soil, light is detected by

at least three classes of photoreceptors in *Arabidopsis* and the seedling proceeds with photomorphogenesis. This is characterised by inhibition of hypocotyl extension, straightening of the apical hook, cotyledon expansion, chloroplast development and de-etiolation (Hart, 1988).

The interaction between red and blue light in potentiating the inhibition of hypocotyl elongation in response to blue light and in anthocyanin accumulation is not a new concept. In 1963 Mohr and van Nes reported that a very small induction of anthocyanin is obtained by red light pulses alone, but that a white light pretreatment results in a much higher responsiveness to the red or far red pulses in buckwheat (*Fagopyrum esculentum*) seedlings. The peak of a rough action spectrum of the potentiating light was in the blue range (action was also seen in the far red range) (Mohr, 1980). Milo (*Sorghum vulgare*) seedlings were treated with three hours of blue light prior to either a five minute pulse of red light, far red light or a far red followed by a red pulse. The plants with a terminal red light exposure accumulated a greater amount of anthocyanin than the plants exposed to a terminal far red light pulse (Drumm and Mohr, 1978).

More recently, Ahmad and Cashmore (1997) reported that CRY1 required low levels of PHYA or PHYB in the active Pfr form to mediate blue light inhibition of hypocotyl length and anthocyanin accumulation. Phytochrome A activity affects hypocotyl growth mediated by phytochrome B Pfr in light grown seedlings (Casal, 1996).

Work by Osterlund and Deng (1998) investigated the accumulation of GUS-COP1 in hypocotyl nuclei in PHYA, PHYB and CRY1 photoreceptor over-expressers and loss of function mutants in various light regimes. Their data correlates with that of Ahmad and Cashmore (1997), further indicating that interaction between phytochromes A, B and cryptochrome 1 regulates hypocotyl extension inhibition. The means of hypocotyl length extension inhibition is regulated by the exclusion of COP1 from the nucleus. In the dark COP1 accumulates in the nucleus and in the light the nuclear exclusion is

dependent mainly on phytochrome A in far red light, PHYB in red light and cryptochrome 1 in blue. There does, however, appear to be some involvement of PHYA in blue light induced GUS-COP1 nuclear exclusion and of CRY1 in far red GUS-COP1 nuclear exclusion (Osterlund and Deng, 1998).

CRY2 and PHYB antagonistically regulate flowering time in *Arabidopsis* via the flowering time gene CONSTANS (CO) (Guo *et al.*, 1998). CRY2 acts as a positive regulator of CO, the increased *CO* gene expression leads to early flowering. *cry2* plants grown under either continuous blue or continuous red light have normal flowering times. If *cry2* is grown under red and blue light together, the mutant plants take longer to flower. *phyB* can suppress the late flowering phenotype of *cry2* plants in blue plus red light. *cry2* cannot, however, suppress the early flowering time of *phyB* plants in red light. Further study of the interaction of phytochromes A and B and CRY1 has been made utilising the availability of the single, double and triple mutants. Neff and Chory (1998) ascertained that in seedlings, PHYA and PHYB act as blue light photoreceptors, and that cry1 is still active in blue light in a *phyA phyB* background. Amongst other observations, they also noted that PHYB and CRY1 modulate anthocyanin accumulation in far red light, despite PHYA being the major photoreceptor for anthocyanin accumulation in seedlings in far red light. Casal and Mazzella (1998) also utilised the double and triple mutants to investigate the interaction of these photoreceptors in the regulation of hypocotyl elongation under various light regimes. They discovered that there is an element of conditional synergism between PHYB and CRY1 dependent on the light conditions present. When seedlings were grown in prolonged blue light supplementing a red light background there was no synergism between CRY1 and PHYB. When blue light was only supplementing far red light for a short time PHYB and CRY1 acted synergistically. This was thought to be due to the combined effect of a reduced PHYB Pfr (in far red light) combined with a limited CRY1 input (only 3 hours in a day) (Casal and Mazzella, 1998).

Another example of phytochrome interaction with other photoreceptors

is that of the phytochrome(s) enhancement of the phototropic response. A red light pretreatment of *Arabidopsis* seedlings leads to a 2 fold greater phototropic curvature response to a subsequent blue light irradiation (Janoudi and Poff, 1991). Phytochrome(s) act as positive regulator(s) for phototropism (Liscum and Briggs, 1996). Further work by Parks *et al.* (1996) indicated that the positive regulation of the phototropic response by a red light pretreatment is in fact mediated mainly by PHYA, though there may be some minor involvement of PHYB.

Liscum and Briggs (1996) showed an interaction between the phytochromes and the phototropic response, where one or more phytochrome(s) act as a positive regulator of the phototropic response. This phototropic response has further been shown to be increased by the cryptochrome photoreceptors (Ahmad *et al.*, 1998a; Christie *et al.*, 1998).

Parks, *et al.* (1996), showed that a red light pulse given prior to the blue light phototropic stimulus increased the curvature in wild type but not in *phyA* mutants, indicating the involvement of PHYA in mediating phototropism. Casal and Boccalandro (1995) showed that PHYA interacts synergistically with the blue light photoreceptor involved in phototropic responses but not with the blue light photoreceptor involved in controlling hypocotyl extension growth.

Although it is now known that cryptochromes 1 and 2 are involved in the phototropic response in a redundant fashion, it was only after the double null mutant was made that any effect of the cryptochromes was seen. The single *cry* mutants were not affected in phototropic response (Ahmad *et al.*, 1998a). Chory *et al.* (1996) suggested there may be an activation of a shared signal transduction pathway by any of PHYA, PHYB or CRY1, as there seem to be a number of overlapping roles, but that specific responses could be mediated by components which interact specifically with the different photoreceptors.

Not only are there interactions between the photoreceptors themselves, evidence of interaction with different regulatory systems has been observed. PHYA, CRY1 and CRY2 are involved in interactions with circadian rhythms

(Somers *et al.*, 1998; Zhong *et al.*, 1997). Zhong *et al.* (1997) investigated the role of PHYA and CRY1 photoreceptors in the damping of the circadian oscillation of a catalase, CAT3. Both PHYA and CRY1 were required for *Arabidopsis* CAT3 to damp to the continuous high level seen in the wild type in response to extended continuous dark conditions. It is not clear whether the involvement of the two photoreceptors is due to a common signal transduction component required to cause the damping, whether one signalling pathway potentiates the other or if a synergistic effect is taking place between the interaction of the two pathways or to another cause.

1.4 Photoreceptor Signal Transduction Components

Photoreceptors must be connected to the appropriate response for a plant to function effectively. The means of signal transduction from stimulus (light detection at the photoreceptor) to response (gene expression, developmental regulation) is far from clear. Mutants in signal transduction are providing a useful tool to elucidate the components involved in signal transduction.

Mutants altered in photoreceptor mediated responses, microinjection studies and a pharmacological approach have been used to attempt to elucidate some of the signalling pathways downstream of the photoreceptors. Mutating agents such as ethyl methanesulphonate (EMS), gamma rays, or introducing mutation by insertion of T-DNA have been used to create the mutants which were then screened for alterations in response to specific stimuli (reviewed in Barnes *et al.*, 1997; Bharti and Khurana, 1997; Chory, 1993; von Arnim and Deng, 1996). Introduction of putative signalling molecules into cell cultures (Frohnmeyer *et al.*, 1998) or into plant cells by direct injection (Neuhaus *et al.*, 1997; Neuhaus *et al.*, 1993) have been used to try to identify molecules directly involved in signalling downstream from a specific photoreceptor. Further elucidation of signalling mechanisms has been provided utilising a pharmacological approach (Bowler and Chua, 1994; Christie and Jenkins, 1996;

Long and Jenkins, 1998; Noh and Spalding, 1998).

1.4.1 Pharmacological Studies

Dissection of the phytochrome signalling pathway has indicated a role for cGMP in the pathway leading to *CHS* induction, whereas calcium and calmodulin are involved in the regulation of *rbcS* and *CAB* genes (Neuhaus *et al.*, 1993). Investigation of signal transduction in the UV-A/blue and UV-B light regulation of *CHS* expression was undertaken using an *Arabidopsis* cell culture (Christie and Jenkins, 1996). The cell culture shows the same responses to UV-A/blue, UV-B, red and far red light as the mature *Arabidopsis* leaf tissue. *CHS* expression is increased in the cell culture in UV-A/blue and UV-B light but no increase is seen after exposure to red or far red light. Introduction of inhibitors of calcium channels resulted in inhibition of *CHS* expression in the cell culture in both UV-A/blue and UV-B light, indicating a role for calcium in the signal transduction pathway. cGMP is not involved in the induction of *CHS* expression in the cell culture. Both UV-B and UV-A/blue inductive pathways are inhibited by protein kinase and phosphatase inhibitors, indicating a role for reversible protein phosphorylation. These inhibitor studies have also revealed a difference between the UV-A/blue and UV-B light signal transduction pathways. Calmodulin is involved in UV-B light induction of *CHS* expression but not in UV-A/blue light induction (Christie and Jenkins, 1996). The calcium requirement of UV/blue light to induce *CHS* expression was further investigated by Long and Jenkins (1998). The electron acceptor, ferricyanide and the flavoprotein antagonist diphenylene iodonium (DPI), both acted to inhibit *CHS* expression in UV-A/blue and UV-B light. The hypothesis presented is of early events in UV-A/blue and UV-B light mediated signal transduction pathways initiated via plasma membrane located redox activity. This redox activity results in a hypothesised increase in cytosolic calcium by release from an intracellular store which is necessary for *CHS* expression (Long and Jenkins, 1998).

Noh and Spalding (1998) have reported that the blue light activation of an anion channel in the plasma membrane is involved in a rapid inhibition of hypocotyl elongation in dark grown *Arabidopsis* seedlings and in anthocyanin accumulation. The rapid hypocotyl inhibition, which takes place within 30 seconds of blue light irradiance, was also seen in the *hy4* mutant, and thus is not mediated by CRY1, though a later more permanent hypocotyl inhibition response (observed after about 8 hours) is mediated by CRY1. The pharmacological studies presented indicate that while blocking the anion channel with 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) does not lower the levels of the gene products involved in the anthocyanin biosynthetic pathway, either at the mRNA level or at the protein level, there is a decrease in anthocyanin accumulation in seedlings treated with NPPB. Feeding studies indicate that *tt5* mutants, which lack chalcone isomerase (CFI), produce greater than wild type levels of anthocyanin when treated with NPPB and supplied with naringenin, the product of the biosynthetic reaction that CFI catalyses. This indicates that the rate limiting step prevented by the addition of NPPB is occurring further upstream than CFI. Feeding wild type plants with phenylalanine in the presence and absence of NPPB resulted in a lower level of anthocyanin accumulation in the presence of NPPB. It was concluded that NPPB inhibited inclusive steps between PAL and CFI. The anthocyanin accumulation in *hy4* plants in the presence of NPPB and sucrose was less than that seen in the wild type under the same conditions. Noh and Spalding suggest that the inhibitory effect of NPPB and the *hy4* mutation are additive and wild type anthocyanin accumulation in blue light requires both a HY4 mediated increase in biosynthetic enzyme transcription and the anion channel pathway mediated increase in activation of enzyme activity upstream of CFI.

1.4.2 Screening for Mutants Altered in Transduction of Photoreceptor Signals

Several types of screens have been used in an attempt to identify light signalling mutants. In some cases visible responses to various light regimes

have been used to isolate mutants (e.g. Koornneef *et al.*, 1980; Liscum and Briggs, 1995), in others reporter genes have been used to monitor the expression of genes (e.g. Jackson *et al.*, 1995; Li *et al.*, 1995).

A number of different reporter genes have been used: the ß-glucuronidase (GUS) gene (Jackson *et al.*, 1995); the firefly luciferase gene (Millar *et al.*, 1995); a gene conferring resistance to hygromycin (Li *et al.*, 1994); and the alcohol dehydrogenase (ADH) gene (Li *et al.*, 1995). The *tms2* gene has also been used to introduce a selectable marker into *Arabidopsis* (Karlin-Neumann *et al.*, 1991). Seedlings containing the *tms2* gene (from *Agrobacterium tumefaciens*) have retarded growth in the presence of concentrations of auxin amides which do not significantly affect the growth of wild type seedlings. So if fused to a light dependent promoter, mutants in the signal transduction pathway could be isolated.

Jackson *et al.* (1995) isolated *icx1*, an over-expressing *CHS* mutant, by screening mutated plants containing a *CHS-GUS* fusion for altered levels of GUS activity. A luciferase gene construct was used to isolate a mutant altered in circadian cycling patterns (Millar *et al.*, 1995). By imaging the bioluminescence of M2 seedlings, the luminescence levels could be used as a screen.

Work has been done using a *CAB* promoter fused to various reporter genes. For instance, the *gun* (genome uncoupled) mutants were isolated by Susek *et al.* (1993). Two reporter genes fused to *CAB3* promoters were used to transform *Arabidopsis*. Mutagenised plants were treated with the herbicide norflurazon to photooxidise the chloroplasts in bright light and grown on hygromycin. The transformed plants carried an *hph* gene (conferring hygromycin resistance) driven by *CAB3*. Only those plants with *CAB3* expression which was not dependent on chloroplast gene expression (which is normal in wild type) would survive.

Li *et al.* (1995) have developed a positive screening technique for mutants altered in their response to light. It is dependent on the *Arabidopsis*

alcohol dehydrogenase gene, which converts allyl alcohol into a toxic product, aldehyde acrolein. In the *Arabidopsis* Bensheim R002 line, containing a null mutation in the *ADH* gene, they introduced a construct which contained two reporter genes, *GUS* and *ADH* genes fused to the *CAB3* promoter. After mutation, it was possible to select for mutants which express low levels of *CAB3* by adding allyl alcohol to the seedlings. Those plants with low expression of *CAB3* converted less of the allyl alcohol into the toxic aldehyde acrolein, and therefore survived the treatment. Cytosine deaminase (CD) can also be utilised as a negative selection agent. Introduction of the CD gene, which is not normally found in higher plants, allows for selection on 5-fluorocytosine (5-FC). CD can convert 5-FC into a toxic compound, 5-fluorouracil (5-FU). Serino and Maliga (1997) introduced *codA* (the CD gene sequence) into the tobacco plastid genome to select for mutants altered in nuclear genes affecting plastid gene expression.

1.4.2.1 Photomorphogenic Mutants

Arabidopsis mutants which fail to follow the appropriate photomorphogenic developmental pathway have provided information about the signal transduction components involved in mediating these changes. *De-etiolated* (*det*) and *constitutive photomorphogenic* (*cop*) mutants were isolated due to the mutants failing to follow the skotomorphogenic growth pathway in the absence of light. The *cop* and *det* mutations affect the signal transduction pathway in such a way that the mutant plants no longer respond to darkness with skotomorphogenic growth. Instead, when the *cop* and *det* seedlings are germinated in the dark they behave to an extent as if they are light grown. The *det* and *cop* mutants have short hypocotyls, open and enlarged cotyledons which contain chloroplast-like differentiation of plastids and cell type differentiation (Deng and Quail, 1992). The *fusca* mutants had been isolated due to high anthocyanin accumulation in embryos or seedlings. It was noted that some of the *fusca* seedlings had the same morphology when grown in either

light or darkness (Miséra *et al.*, 1994). Six *fusca* mutants were identified as alleles of *cop* and *det* mutants. As a result of these screens for mutants altered in photomorphogenesis, ten *COP/DET/FUS* genes have been isolated (Kwok *et al.*, 1996). These ten genes are required for normal repression of photomorphogenesis in the dark. The *COP/DET/FUS* genes act to repress chloroplast development in the dark and in non-photosynthetic tissue.

Not all the mutants isolated as photomorphogenic mutants were involved in the direct transduction of light signals. *det2* (Chory *et al.*, 1991) was found to be a mutant in the brassinolide biosynthetic pathway. This does point to an interaction between plant hormones and light regulated development (Chory and Li, 1997).

COP genes involved in photomorphogenesis include *COP1/FUS1, COP8/FUS8, COP9/FUS7, COP10/FUS9, COP11/FUS6* (Kwok *et al.*, 1996). While the *cop* mutants are altered in the developmental switch from dark to light growth, the lethality of the severe mutations indicates a role for the *COP* genes during vegetative growth (Karniol *et al.*, 1999; Mayer *et al.*, 1996; Miséra *et al.*, 1994). COP9 has been cloned and it has been shown to be a component of a signalling complex (Wei *et al.*, 1994). The complex includes COP8, COP9 and COP11 (Chamovitz *et al.*, 1996; Staub *et al.*, 1996; Wei *et al.*, 1994). The disruption of any of these proteins by mutation causes the visible phenotype of the *cop* mutants. Wei *et al.* (1994) reported that COP9 is part of a light stable complex of greater than 560 kDa. In etiolated seedlings some of this complex is shifted to a higher molecular mass. This multi-subunit complex present in darkness, disperses within five minutes of light exposure. The disruption of this complex results in a photomorphogenic growth pattern in the dark. The COP8, COP9 and COP11 proteins are thought to be involved in the multi-subunit complex, because, in the *cop8* and *cop11* mutants, no multi-subunit complex can be detected. Recently, a further component of the COP9 complex has been cloned. The 27 kDa subunit of the complex was cloned and is encoded by *FUSCA5* (Karniol *et al.*, 1999). FUS5 could be phosphorylated *in vitro* in the

presence of *Arabidopsis* plant extracts. The COP9 complex is conserved in animal systems and the human COP9 complex is referred to as the COP9 signalosome (Karniol *et al.*, 1999). It has been suggested that the human and plant COP9 complexes both take the name COP9 signalosome (Wei and Deng, 1999).

The *cop1* mutant led to the cloning of a protein which was found to play a pivotal role in the regulation of photomorphogenesis (Deng *et al.*, 1992). COP1 is made up of three recognisable structural domains; the zinc binding motif, the putative coiled-coil region, and a domain of multiple WD40 repeats. Investigation of a series of mutations in COP1 (McNellis *et al.*, 1994) indicates that a number of mutations in these structural domains can cause varying phenotypes, dependent on where the mutation is and the nature of the disruption. A mutation resulting in a protein containing only the N-terminal 282 amino acids of COP1 (encoding the zinc binding and coiled-coil domains) shows only a weak phenotype, however, if a full length COP1 protein is present with a mutation in the domain of the WD40 repeats this masks any of the N-terminal activity. Expression of the N-terminal 282 amino acids of COP1 in *Arabidopsis* plants (containing wild type COP1 protein) is sufficient to induce pleiotropic effects similar to that of the *cop1* mutation (McNellis *et al.*, 1996). The putative coiled-coil domain and the WD40 repeats of COP1 show homology with the *Drosophila* dTAF$_{II}$80. dTAF$_{II}$80 is a component of the RNA polymerase II transcription apparatus (Dynlacht *et al.*, 1993). Further homology with the WD40 repeat was found with the yeast protein TupI, which is thought to be a global repressor of transcription (Keleher *et al.*, 1992).

Localisation of COP1 was visualised using a COP1-GFP protein. In dark grown plants COP1 was found to be localised within the nucleus. Upon irradiation, the COP1 protein was relocalised into the cytosol. COP1-GFP took 36 hours for full exclusion from the nucleus. Due to the time difference between nuclear exclusion and the much shorter time for the initiation of photomorphogenesis, COP1 nuclear exclusion is suggested to be a method of

maintaining photomorphogenesis, rather than a mechanism for initiating it (von Arnim and Deng, 1994). Osterlund and Deng (1998) utilised photoreceptor mutants and photoreceptor over expressing plants to visualise nuclear abundance of GUS-COP1 in different light qualities. It was concluded that the three photoreceptors investigated, PHYA, PHYB and CRY1 played a role in far-red, red and blue light mediated COP1 nuclear exclusion, respectively. An interacting protein, the COP1 Interacting Protein (CIP1) may play a role in nuclear localisation of COP1 (Matsui *et al.*, 1995).

COP1 interacts directly with HY5 (Ang and Deng, 1994; Ang *et al.*, 1998). *hy5* was isolated as a long hypocotyl mutant (discussed in detail in Section 1.4.2.2) A further protein found to interact with COP1 is the CIP7 protein (Yamamoto *et al.*, 1998). CIP7 (COP1 interaction protein 7) appears to interact with COP1 via a coil motif. Disruption of this protein by introduction of antisense sequence results in reduced expression of *CHS* and also of genes involved in chloroplast development. Unlike *hy5*, no effect was seen in hypocotyl elongation in the CIP7 antisense plants (Yamamoto *et al.*, 1998). CIP7 and HY5 may be components in signalling pathways affecting different subsets of responses downstream from COP1.

The *det1* mutant of *Arabidopsis* shows constitutive photomorphogenesis (excluding chlorophyll synthesis) when grown in the dark. The light grown *det1* phenotype includes an increase in anthocyanin accumulation and reduced hypocotyl elongation (Chory and Peto, 1990; Chory *et al.*, 1989). As yet, it is not known how the *DET1* gene functions as there have been no indications of direct DNA binding. DET1 contains a bipartite nuclear localisation signal, and is mainly found in the *Arabidopsis* nuclei (Pepper *et al.*, 1994). It has been suggested that transcriptional regulation by DET1 may be achieved by higher level organisation such as chromatin structural regulation (Chory *et al.*, 1996).

A homologue of the *Arabidopsis DET1* gene has been found in tomato (Mustilli *et al.*, 1999). The tomato *high pigment-2* (*hp2*) mutant has increased anthocyanin accumulation, increased chlorophyll accumulation and reduced

hypocotyl lengths when grown in the light, but no obvious dark phenotype. The *hp2* mutant was found to be altered in a gene with 81.3% protein sequence similarity (74.8% identity) to that of DET1, the gene was named *TDET1*. Despite the mutation in *TDET1*, the tomato homologue of the *Arabidopsis DET1* gene, the phenotypes in tomato and *Arabidopsis* are different, perhaps due to a different system for photoreceptor signal transduction.

Not only do *cop1*, *det1* and *cop9* mutants show altered photomorphogenic responses, they also have altered gene expression from that of wild type. Light responsive gene expression is altered; *CAB* is underexpressed and *CHS* is overexpressed in the *cop1*, *cop9* and *det1* mutants, compared to wild type plants. *cop1* and *cop9* additionally show increased expression of genes normally induced by hypoxia (Mayer *et al.*, 1996). Thus there is a role for COP1, COP9 and DET1 in a wider sphere of regulatory control than just that of light dependent signal transduction. DET1 is involved in tissue specific regulation of at least one promoter. The chloroplast blue light-responsive promoter undergoes transcriptional repression by DET1 in a tissue specific and a developmental manner (Christopher and Hoffer, 1998).

Crosses between the *cop1* and the *det1-1* mutants and *cop1* and *hy* mutants (Ang and Deng, 1994) imply that DET1 and COP1 act in the same pathway and that the *cop1* mutations (unless weak) can suppress those of the *hy1*, *hy2*, *hy3* and *hy4* mutations. The results would seem to point to COP1 acting downstream from DET1 and the phytochromes and a blue light receptor. The COP/DET/FUS proteins appear to act downstream from PHYA, PHYB, and HY4, as the double mutants display the phenotype of the *cop/det/fus* mutants (Kwok *et al.*, 1996).

1.4.2.2 *hy5* Mutant

In the same screen which isolated the phytochrome deficient mutants *hy1* and *hy3* (*phyB*) the *hy5* photomorphogenic mutant was also isolated (Koornneef *et al.*, 1980). *hy5* was isolated as a long hypocotyl mutant due to its

lack of hypocotyl inhibition under various light conditions. *hy5* does not show inhibition of hypocotyl elongation mediated by red, far-red or blue light and thus HY5 is thought to act downstream of the red, far-red and blue light photoreceptors in this response. *hy5* has normal amounts of spectrally active phytochromes (Koornneef *et al.*, 1980). Anthocyanin accumulation is decreased in *hy5* mutants in high white light compared to wild type plants (Chory, 1992). The *hy5* mutant does not show the PHYA mediated block in greening after exposure to far-red light (Barnes *et al.*, 1996a), indicating that HY5 does not act downstream of PHYA in the regulation of de-etiolation but that it is involved in the regulation of a separate pathway leading to hypocotyl elongation inhibition. Oyama *et al.* (1997) investigated the role of HY5 in roots and hypocotyl, they concluded that HY5 was involved in the gravitropic response of roots, regulation of root hair length and that the *hy5* mutation affected greening and cell proliferation. The overexpression of HY5 reduces the formation and elongation of lateral roots (Okada *et al.*, 1998) suggesting a role for HY5 as a negative regulator of lateral root formation and elongation.

The *Arabidopsis HY5* gene has been genetically defined as a positive regulator of photomorphogenesis that is negatively regulated by COP1 (Ang *et al.*, 1998). It has been shown to encode a basic leucine zipper (bZIP) type of DNA binding protein (Oyama *et al.*, 1997). Allele specific interactions between *cop1* mutants and *hy5* mutants indicate that there is direct physical interaction between the two gene products (Ang and Deng, 1994).

The basic region of HY5 has an identical sequence to that of the soybean STF1A which preferentially binds to TGACGT core DNA sequences (Oyama *et al.*, 1997). Oyama *et al.* also suggest that as HY5 has a consensus sequence for phosphorylation by casein kinase II (CK2), that phosphorylation of this putative CK2 site may enhance DNA binding, as is seen in the *Arabidopsis* GBF1 bZIP protein. Chattopadhyay *et al.* (1998) report that HY5 is constitutively nuclear localised and that it can bind specifically with G-box DNA sequences. G-box sequences are found in light responsive elements (LRE) which are found

in promoters of light regulated genes. The HY5 fusion protein tested was not found to bind to either of the light regulatory sequences elements, GATA or GT1 tetramers. Ang and Deng (1994) initially reported that HY5 interacts with COP1 in the nucleus. This conclusion was reached due to interactions between different mutant alleles. Ang *et al.* (1998) further dissected the interaction between COP1 and HY5 and found that the N-terminal 77 amino acids are required to interact with COP1. Deletion of the N-terminus of HY5 abolishes its ability to interact with COP1 as ascertained by the yeast two-hybrid system. From this work it was concluded that HY5 contains at least two distinct regions, the N-terminus, where HY5 interacts with COP1 and the putative dimerisation and DNA-binding C-terminus. Overexpression of a truncated HY5 gene, lacking the COP1 interaction domain, in transgenic plants produced plants which were hyper responsive to light. The phenotype included increased anthocyanin accumulation and reduced hypocotyl length. The overexpression of full length HY5 did not cause a similar phenotype, indicating that HY5 is negatively regulated by COP1 (Ang *et al.*, 1998).

Ang *et al.* (1998) further report that accumulation of *HY5* mRNA is modulated in 3 day old seedlings by *COP1*, as the *HY5* mRNA light and dark accumulation was increased in the *cop1-6* mutant, but not in either the light grown *hy2-1* (phytochrome) or *hy4-1* (cryptochrome 1) mutant plants. In roots of mature (30 day old) *cop1-6* and *det1-1* mutant plants *HY5* accumulates to higher levels (five and three times higher, respectively) than is seen in the wild type. Not only *COP1* but also *DET1* seems to play a role in the repression of HY5 accumulation as *HY5* gene expression levels are higher in both *det1* and *cop1* mutants (Oyama *et al.*, 1997). An overview of the HY5, COP1 interactions can be found in Jarillo and Cashmore (1998).

Ang *et al.* (1998) further detail that HY5 can interact directly with the *CHS* promoter and that it is essential for light activation of *CHS*. It is unlikely that HY5 is the only transcription factor able to activate all the light responsive elements, because not all light activated genes contain light regulatory

sequences with a G-box element. The G-box itself is insufficient to confer light regulation of gene expression (Puente *et al.*, 1996), so HY5 may be one of a number of transcription factors able to bind and activate gene expression in response to light. Alternatively HY5 may bind preferentially to a different sequence which was not investigated by Chattopadhyay *et al.* (1998) perhaps the TGACGT core site suggested by Oyama *et al.* (1997), from similarities of HY5 sequence to the soybean STF1A. The binding of STF1A to G-box containing DNA sequences is only weak (Cheong *et al.*, 1998).

1.4.2.3 *transparent testa* Class of Mutants

The *cop/det/fus* mutants show an increased accumulation of anthocyanin as part of their phenotype. A separate set of mutants has been isolated which also affect anthocyanin accumulation, in this case the mutations result in a lack of appropriate anthocyanin accumulation. The *tt* mutants are affected in the brown colouration of the seed coat of *Arabidopsis*. The brown colour is normally a result of anthocyanin and tannin accumulation in the seed testa, the mutants lacking this pigmentation were therefore called *transparent testa* (*tt*) mutants.

A study of the *tt* mutants by Shirley *et al.* (1995) was made in order to investigate the genetics of flavonoid biosynthesis. At least eleven *tt* loci have been isolated. The *tt3*, *tt4*, *tt5* and *ttg* (*transparent testa, glabra*) mutants are also affected in the accumulation of anthocyanins in leaves. The *ttg* mutation has an additional affect on trichome and root hair development. Further differences between the the various *tt* mutations can be seen when the floral organs are compared under UV light. There are differences between wild type and the *tt3*, *tt4*, *tt5*, *tt6* and *ttg* seeds and petals, the differences are consistent with a lack of flavonols in the tissues. Some of the *tt* mutants have been shown to be mutated in genes which act in the flavonoid biosynthetic pathway. It is this pathway which leads to anthocyanin biosynthesis and the production of many other flavonoids.

1.5 Flavonoids

Over 3000 flavonoids have been isolated. In some plant species, flavonoids are important in flower pigmentation, fertility and in legumes, the release of flavonoids is involved in the specificity of the host-bacteria interactions of the plant and the nitrogen fixing bacteria (reviewed inShirley, 1996). In maize and petunia, mutants that are flavonoid deficient are sterile (Coe *et al.*, 1981; Taylor and Jorgensen, 1992). However, *Arabidopsis* plants with mutations at the *CHS* locus (*tt4* mutants, resulting in plants with no flavonoid accumulation) are fully fertile (Burbulis *et al.*, 1996).

Flavonoids are phenolic compounds which are secondary metabolites, common to all higher plants. Flavonoids are composed of two phenolic rings connected by 3 carbons. It is the degree of oxidation of this 3 carbon unit which is used to classify the flavonoids into the flavonols, flavanones, isoflavanoids and the anthocyanins. Further modification of each subclass results in the massive range of flavonoids (Koes *et al.*, 1994).

Mutants altered in this aromatic secondary product biosynthesis display altered phenotype in response to various fluence rates of UV-B (Landry *et al.*, 1995; Li *et al.*, 1993). Figure 1.5.1 shows the flavonoid biosynthetic pathway, and where the enzymes discussed below function in it. It should be noted that *PAL* functions upstream from both flavonoids and sinapic acid ester biosynthesis, both of which compound are implicated in UV-B protection. The *CHS* enzyme is the first step which commits to flavonoid accumulation.

Sunlight contains light qualities required by the plant for energy harvesting, positional cues and information about surrounding plant growth (via reflection and absorption by neighbouring plants altering the light quality). Sunlight also contains damaging high energy ultraviolet (UV) rays. UV light can be very damaging to plants. UV-B (280-320 nm) can cause damage to DNA, RNA and proteins (Ballaré *et al.*, 1996; Li *et al.*, 1993). A summer annual, *Datura ferox* L. grown in full sunlight in Buenos Aires had greater DNA damage than those grown under UV-B filters. Damage was measured by the DNA damage

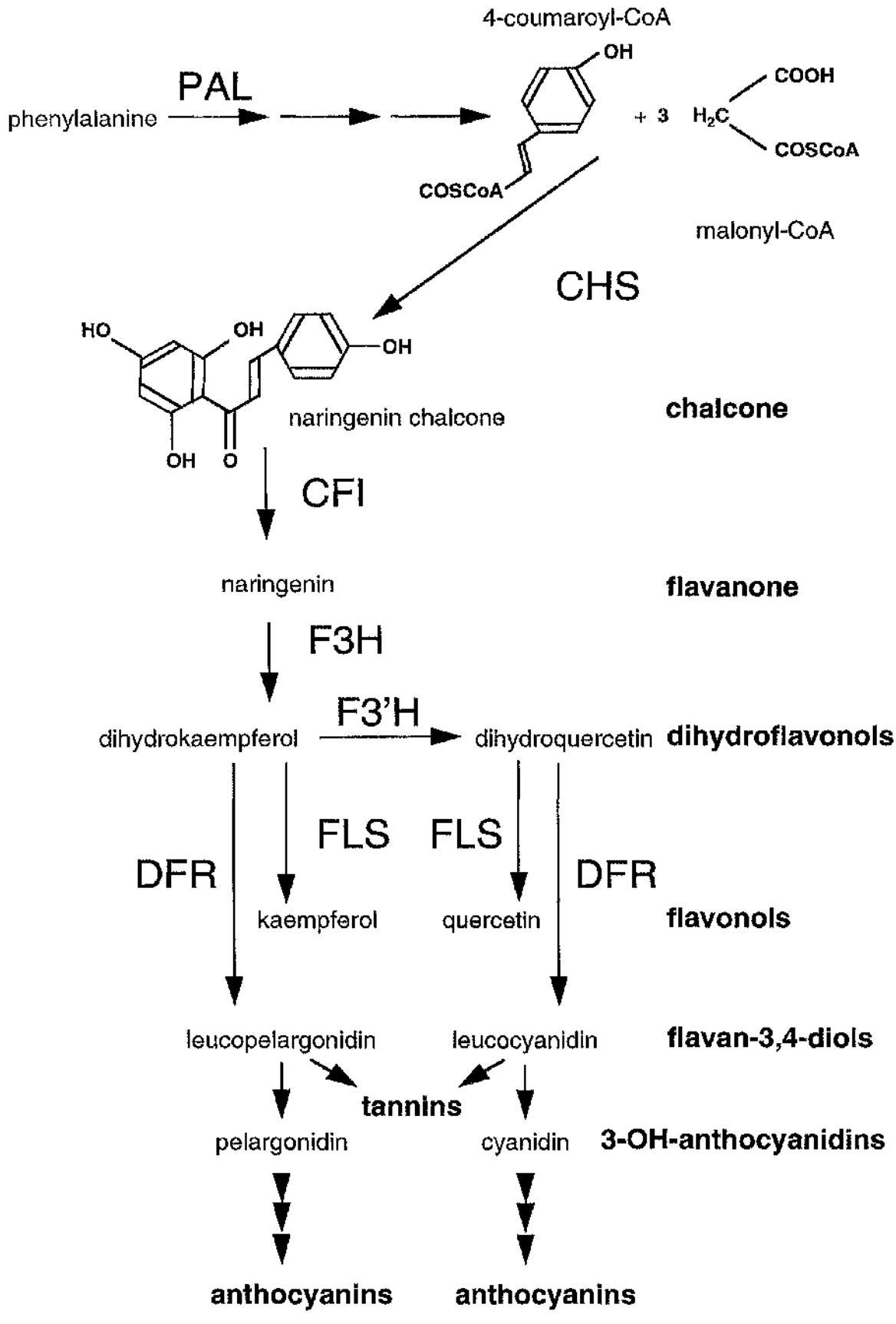


Figure 1.5.1 The flavonoid biosynthetic pathway

product cyclobutane-pyrimidine dimer (CPD) (Ballaré *et al.*, 1996). Mutants altered in their ability to repair DNA damage (in the forms of CPD and pyrimidine-pyrimidinone (6-4) dimers) are more sensitive to UV-B light than wild type plants (Britt *et al.*, 1993; Landry *et al.*, 1997). UV-B potentiates accumulation of UV-B screening compounds in field grown plants (Robberecht and Caldwell, 1986) and is involved in synergistic increases of biosynthetic enzymes leading to flavonoid accumulation (see Section 1.7.2).

1.5.1 Flavonoids play a role in UV protection

Flavonoids accumulate in the epidermis, it is thought that they act as a form of "sun screen" for the plant, absorbing potentially damaging UV light at the leaf surface and preventing its penetration into the mesophyll (Li *et al.*, 1993). In one study template plasmid DNA was irradiated with UV-B light in the presence or absence of flavonoids (Kootstra, 1994). The presence of the flavonoids, naringenin and rutin, or an extract from apple skin prevented DNA damage accumulation. Lois (1994) reports that in *Arabidopsis*, an increase in flavonoids can be seen localised in the epidermis, correlating to areas treated with UV-B irradiation. The *ferulic acid hydroxylase* 1 (*fah1*) mutant is able to accumulate UV-absorbing flavonoids but is unable to synthesise sinapate esters. The *tt4* (*CHS* null mutant) and *tt5* (*CFI* null mutant) are also altered in their ability to produce flavonoids and sinapate esters (Li *et al.*, 1993). The *tt4* mutant shows a lack of the flavonoid kaempferol but an increased level of sinapic acid esters compared to the wild type plants. The *tt5* mutant lacks detectable kaempferol and also has reduced sinapate esters. The *fah1* mutant suffers more UV-B injury than the wild type, as do the *tt4* and the *tt5* mutants. This indicates that both flavonoids and sinapate esters are important in UV protection (Landry *et al.*, 1995; Li *et al.*, 1993; Lois, 1994). UV-B light induced damage was greater in *Zea mays* plants without the regulatory genes for anthocyanin biosynthesis than in those containing anthocyanin (Stapleton and Walbot, 1994). Anthocyanins accumulate in response to UV-B light, and while

the absorption maxima of anthocyanins is in the region of 530 nm, they can absorb UV-B when esterified to cinnamic acid (Taylor *et al.*, 1997).

1.5.2 Flavonoid biosynthesis

Flavonoid biosynthesis is under temporal control, with the expression level of several flavonoid biosynthetic genes peaking in 3 day old *Arabidopsis* seedlings grown in continuous white light (Kubasek *et al.*, 1992). In *Arabidopsis* the increase in flavonoid biosynthetic enzymes in white light is due, at least in part, to a blue light receptor. In dark or red light grown *Arabidopsis* seedlings additional blue light caused an increase in four flavonoid biosynthetic genes, phenylalanine ammonia-lyase 1 (*PAL1*), chalcone synthase (*CHS*), chalcone isomerase (*CFI*), and dihydroflavonol reductase (*DFR*). The order of induction of the genes is the same as the order of biosynthetic steps in the biosynthetic pathway. The CHS and CHI proteins are seen to increase in the same manner as the mRNA transcripts, though the proteins are detectable for longer than the transcripts (Cain *et al.*, 1997). Kubasek *et al.* (1992) also reported that the induction of the flavonoid biosynthetic genes increased above that in white light with the addition of UV-B light. In the experimental set up used, plants were exposed to UV-B light and also blue light or blue light alone, to compare the difference between the two treatments. The effect of UV-B light was taken as the difference between the two treatments. This may, however, have been misleading as Fuglevand *et al.* (1996) have shown that there is a synergistic effect seen on the induction of *CHS* in plants grown in UV-B and blue light together (see Section 1.7.2). It is perhaps more likely that in the study by Kubasek *et al.* had the seedlings been exposed to UV-B light alone, the increase in the flavonoid gene expression levels would not have been so great as was detected in the presence of both blue and UV-B light. In soybean seedlings *CHS* expression was increased after exposure to UV-B light (Shimizu *et al.*, 1999)

Anthocyanin accumulation can also be seen in the reddish pigment accumulation when plants are exposed to conditions which increase flavonoid

biosynthetic pathway enzyme activity. These conditions include high white light, cold, high sugar/low nitrate availability, addition of phenylpropanoid pathway intermediates and UV-A light and UV-B light (Jackson and Jenkins, 1995; Leyva *et al.*, 1995; Loake *et al.*, 1991; Lois, 1994). In addition, inorganic phosphate (Pi) starvation results in increased anthocyanin accumulation (Trull *et al.*, 1997). Bariola *et al.* discovered that antisense expression of two S-like ribonucleases, *RNS1* and *RNS2* which normally increase upon Pi starvation, resulted in increased anthocyanin accumulation (1999).

In maize (*Zea mays*) Myb homologous oncoproteins, C1 and Pl, and the basic-helix-loop-helix protein, R and B, control the expression of the structural genes involved in anthocyanin biosynthesis (Goff *et al.*, 1992; Lesnick and Chandler, 1998). Investigation of C1 and B binding in the maize *a1* and *a2* anthocyanin biosynthetic enzyme promoters indicate that the binding of C1 to the promoter is required for activation of both *a1* and *a2*. Conserved sequences named ARE's (anthocyanin-regulatory element) are present in the maize anthocyanin biosynthetic enzyme promoters *a1*, *a2*, *bz1* and *bz2*. These sequences are not absolutely required for C1 and B mediated activation and are separate from the C1 binding sequences though are in the same region in the *a1* and *a2* promoters (Lesnick and Chandler, 1998).

1.6 Chalcone Synthase

CHS is the first committed step in the flavonoid biosynthetic pathway, leading to the production of flavonoids and anthocyanins (see Section 1.5). Not only is chalcone synthase (*CHS*) the first enzyme in flavonoid biosynthesis, but also in *Arabidopsis*, *CHS* gene regulation is an ideal model system for investigation of UV/blue light signal transduction. This is for a number of reasons, including that in *Arabidopsis* there is only a single *CHS* gene (Burbulis *et al.*, 1996; Feinbaum and Ausubel, 1988), unlike in other species where gene families exist. The genetic approach is also suitable for isolation of mutants altering *CHS* expression. The single *CHS* gene present in *Arabidopsis*, allows

mutations affecting *CHS* expression to be directly observed and not masked by gene family members as would be the case in other plant species.

CHS is expressed in the epidermis and vascular tissue where anthocyanins and flavonoids accumulate. In wild type *Arabidopsis* plants, *CHS* gene expression is under strict developmental control. In mature plants *CHS* is known to increase in response to irradiation by blue and UV light (Jenkins *et al.*, 1995), sugar (Tsukaya *et al.*, 1991) and cold (Leyva *et al.*, 1995). In leguminous plants *CHS* is expressed in response to fungal elicitors (Loake *et al.*, 1991) but in *Arabidopsis*, while *PAL* is induced, *CHS* is repressed by fungal elicitor treatment (Logemann *et al.*, 1995). When plants are grown under low fluence rate white light (about 20 μmol m^{-2} s^{-1}) at room temperature there is no significant induction of *CHS* mRNA transcripts.

The phytochromes absorb in the blue and UV-A region of the spectrum, however, Batschauer (1996) concluded that *CHS* regulation in *Arabidopsis* seedlings is independent of the phytochromes. Using a combination of transgenic approach and utilising the availability of phytochrome null mutants, Batschauer *et al.* (1996) reported that in the absence of phytochrome A and phytochrome B, *CHS* is still regulated by UV-A and blue light, though a slight reduction in *CHS-GUS* expression was seen in the *phyA* mutant. Further investigation of the degree of overlap between PHYA, PHYB and CRY1 mediated responses was made utilising the null mutants for these photoreceptors (Neff and Chory, 1998; Poppe *et al.*, 1998). The studies were of seedlings and the conclusion of both studies was that there is some interaction of phytochrome and cryptochrome 1 in de-etiolation responses, but that this is dependent on the light fluence rate (Poppe *et al.*, 1998) and light quality (Neff and Chory, 1998). Neff and Chory (1998) concluded that in 5 day old seedlings anthocyanin accumulation in blue light required both PHYA and CRY1, but not PHYB as the *phyA* and *cry1* mutants lacked anthocyanin accumulation, and that PHYA was required in far red light anthocyanin accumulation. (Poppe *et al.*, 1998) also found a PHYA requirement for anthocyanin accumulation in far red

light. In contrast to the work of Neff and Chory (1998), Poppe *et al.* (1998) found that PHYB was also involved in the regulation of anthocyanin accumulation in blue light. The differences between the phytochrome requirement seen in the work of Neff and Chory (1998) and Poppe *et al.* (1998) and that of phytochrome independence of Batschauer (1996) may be a function of the different products investigated. Whereas Batschauer investigated *CHS* expression (via a *CHS-GUS* transgene) the other studies focused on an end product (anthocyanin) mediated by *CHS* expression.

1.6.1 *CHS* Developmental Regulation

Kubasek *et al.* (1992) and Kaiser *et al.* (1995) investigated the role of different light qualities on flavonoid biosynthetic gene expression in etiolated seedlings. In 6 day old etiolated *Arabidopsis* seedlings *CHS* expression was activated by far-red light but this far-red responsiveness was lost in 8 and 10 day old seedlings. This is different to the case in mustard, where both red light and far-red produced an increase in *CHS* levels in 6 day old seedlings (Batschauer *et al.*, 1991). By the time *Arabidopsis* seedlings are 10 days old UV-A light is more effective in the induction of *CHS* expression (Kaiser *et al.*, 1995). The results that Kaiser *et al.* (1995) presented were somewhat different to those presented by Kubasek *et al.* (1992). Whereas Kaiser *et al.* found that *CHS* expression was induced by far-red and UV-A light, but not by blue, Kubasek *et al.* found that their seedlings responded to blue light but not UV-A and far-red. Both sets of workers found that there was the same age dependent potential for induction of anthocyanin biosynthetic enzymes. This age dependent potential exists in the *Arabidopsis* seedling regardless of the photosynthetic capacity of the seedling or of the stage of cotyledon expansion, as the accumulation potential was constant in seedlings grown either in darkness or in red light (Kubasek *et al.*, 1998). It can only be assumed that the differences in the findings of Kubasek *et al.* (1992) and Kaiser *et al.* (1995) were due to the quality and purity of the light sources available to each group and the fluence rates the seedlings were

subjected to prior to assaying.

1.6.2 *CHS* Promoter

A number of studies have been made with the whole or part of a *CHS* promoter fused to a reporter gene. From these studies, a number of sequences involved in the regulation of the *CHS* gene have been identified. Schulze-Lefert *et al.* (1989a; 1989b) investigated the role of promoter elements controlling *CHS* expression in parsley (*petroselinum crispum*). This work with UV containing white light defined two light responsive units (LRUs) involved in UV regulation of *CHS*. The two LRUs were made up of four *cis*-acting elements which were bound by proteins in response to irradiation. The parsley *CHS* LRU1 contains an ACGT-containing element (ACE^{PcCHS}) and a MYB recognition element (MRE^{PcCHS}) and was found to be sufficient to confer light dependent regulation.

Kaiser *et al.* (1995) further investigated the elements within the mustard *CHS1* promoter which were required for light mediated expression. The conclusion was that Unit 1 is sufficient for developmental regulation, tissue specificity and light mediation of *CHS* in *Arabidopsis*. Unit 1 includes a G-box-like sequence.

Hartmann *et al.* (1998) used *Arabidopsis CHS* promoter deletions to investigate promoter elements involved in light responsiveness to UV-A and UV-B light. The 48 bp region of the LRU^{AtCHS} is both necessary and sufficient to confer light regulation of transcription, mediating both the regulation by UV-A and UV-B light. The two phototransduction pathways appear to regulate transcription factors which interact with common promoter elements. Hartmann *et al.* also noted that the LRU^{AtCHS} is not the sole regulatory sequence in the *Arabidopsis CHS* promoter as there are elements further upstream in the promoter that are able to induce *CHS* transcription in the absence of the LRU^{AtCHS}.

CHS regulation in *Arabidopsis* by UV-A and UV-B light has been shown

to be mediated by separate pathways (Christie and Jenkins, 1996; Fuglevand *et al.*, 1996). CRY1 mediates a significant portion of the *CHS* induction in *Arabidopsis* leaves when they are treated with blue light (Fuglevand *et al.*, 1996; Jackson and Jenkins, 1995) as the *CHS* levels in the *hy4-2.23N* (null cryptochrome) mutant exposed to blue light are much lower than in the wild type control. In UV-A light, the *CHS* transcript levels in *hy4-2.23N* are nearly undetectable. In UV-B light, however, *CHS* transcript levels in the *hy4-2.23N* are equivalent to those of the wild type (Fuglevand *et al.*, 1996). In the double cryptochrome null mutant *cry1cry2* UV-B light mediated *CHS* expression was found to be equivalent to that of the wild type (Valentine, 1998). This indicates that neither cryptochrome 1 nor cryptochrome 2 is mediating the UV-B response.

It is likely that different photoreceptor systems are mediating the *CHS* induction in blue/UV-A and UV-B light. Pharmacological investigation of signalling components involved in transducing the UV-A or UV-B signal to increased *CHS* expression was made by Christie and Jenkins (1996) in a photomixotrophic cell culture. Use of W-7, a calmodulin antagonist, produced an inhibition of *CHS* expression in response to UV-B light induction but not to that of UV-A/blue light. Conversely, Long and Jenkins (1998) have shown that addition of the Ca^{2+}-ATPase inhibitor erythrosin B prevented the UV-A/blue light response but not that mediated by UV-B.

Leyva *et al.* (1995) have presented results which have indicated that in light, cold increases *CHS* expression. Further work in our laboratory has shown that it is blue light which is the light quality responsible for the increase (Clucas, Stewart and Jenkins, unpublished data).

1.6.3 Transcriptional Activators of *CHS*

Transcription factors involved in the regulation of anthocyanin accumulation in maize (*Zea mays*) have been found to be active in other plant species (Lloyd *et al.*, 1992; Quattrocchio *et al.*, 1998). Co-expression of R and C1

(see Section 1.5.2) is required in transgenic *Arabidopsis* to cause an increase in anthocyanin accumulation (Lloyd *et al.*, 1992). Additionally, the *ttg* mutant of *Arabidopsis*, which does not produce anthocyanin (see Section 1.4.2.3) can be restored by introduction of the maize *R* gene (Lloyd *et al.*, 1992).

Plant bZIP transcription factors bind to DNA sequence elements containing ACGT cores (Foster *et al.*, 1994), the flanking sequences affecting binding specificity. The ACGT sequence is then named according to the flanking sequences, giving rise to the G-box, C-box and A-box (Izawa *et al.*, 1993). A further level of regulation is by phosphorylation of the transcription factors, which can lead to altered DNA binding affinity, nuclear localisation and transcriptional activation (Hunter and Karin, 1992). Reduction by antisense expression of *Arabidopsis* CK2, which phosphorylates transcription factors, caused an increase in the level of *CHS* transcript accumulation in the dark and also altered *rbcS* and *CAB* expression in red light (Lee *et al.*, 1999). Plant transcription factors are reviewed by Ramachandran *et al.* (1994).

The G-box binding factor (GBF) binds to the *CHS* promoter as do bZIP factors. A study by Ang *et al.* (1998) reports that the HY5 bZIP transcription factor (Section 1.4.3) can bind directly to the LRE of the *CHS1* gene from mustard. Ang *et al.* note that HY5 is essential for the light activation of the *CHS1* minimal promoter. In white light no expression of *CHS* promoter-GUS reporter construct was seen in the *hy5* mutant. This requirement of HY5 for *CHS* expression indicates a mode of regulation of *CHS* expression by COP1. COP1 is present in the nucleus in the dark and is translocated out of the nucleus by signals downstream from PHYA, PHYB and CRY1 in far-red, red and blue light (Osterlund and Deng, 1998). COP1 recruits HY5 into nuclear speckles and only releases HY5 to interact with DNA on illumination, which results in nuclear exclusion of COP1. Removal of COP1 from the nucleus removes the negative regulation of HY5 which can then interact with the LRU of the *CHS* promoter and result in increased *CHS* expression (Ang *et al.*, 1998).

1.7 Synergistic Regulation of *CHS* Expression

CHS expression can be greatly increased under certain synergistic conditions. To explain synergism, imagine the case where *CHS* expression was increased X amount in response to signal x and Z amount in response to signal z when either signal was present alone. If a plant was then exposed to both signals x and z at the same time, the resultant response could be one of several possibilities. Four possible means of incorporation of two separate signals are i) no increase above either X or Z ii) an additive increase where the resultant signal equalled X+Z iii) a synergistic interaction, where the resultant signal was greater than the sum of X+Z and iv) a negative regulation where the resultant signal is less than X or Z.

A number of synergistic interactions occur to cause an increase in *CHS* expression in plants. These interactions include the red light and blue light interaction which causes a synergistic increase in anthocyanin accumulation and the blue or UV-A light interaction with UV-B light which leads to increased *CHS* expression (Fuglevand *et al.*, 1996).

1.7.1 Red and Blue Light Synergism

The red light and blue light interaction was initially noted due to the observed increase in anthocyanin accumulation in plants exposed to both blue and red light. The effect was termed co-action. Mancinelli *et al.* (1991) treated cabbage and tomato seedlings with blue and red light or with blue and far red light, comparing the anthocyanin accumulation to that of seedlings treated with either red light alone or far red light alone. They found that there was interaction between phytochrome and cryptochrome in mediating the anthocyanin accumulation in response to blue and red or far red light. Work completed by Ahmad *et al.* (1997; 1998b) showed that cryptochrome requires (at least) low levels of active phytochrome for full activity. This interaction between phytochrome and cryptochrome is believed to be that of phytochrome phosphorylating cryptochrome.

1.7.2 Blue/UV-B and UV-A/UV-B Light Synergism

In 1989, Ohl *et al.* reported that in parsley cell culture a blue light treatment was sufficient to produce a signal which could produce an increase in the subsequent induction of *CHS* expression upon UV light treatment (Ohl *et al.*, 1989).

In *Arabidopsis*, blue, UV-A and UV-B light result in accumulation of *CHS* transcript. Fuglevand *et al.* (1996) reported a synergistic effect between blue and UV-B light. Using transgenic plants containing the *CHS* promoter fused to the *GUS* reporter gene, plants were assayed for expression of *CHS* by GUS activity. When plants are exposed to blue light at the same time as UV-B light, the expression of *CHS* transcripts is greater than the additive amount of expression of the single light qualities alone. This synergistic effect is also noted between UV-A and UV-B, implying that at there are at least two signal transduction pathways (UV-A and UV-B, and blue and UV-B) regulating the expression of *CHS*. Blue light produces a stable signal, with synergistic interaction seen if the blue and UV-B light treatments are given sequentially. UV-A does not produce a stable signal, synergism is only seen if the UV-A and UV-B light treatments are concurrent. Fuglevand *et al.* (1996) noted that the blue plus UV-B and UV-A plus UV-B synergistic expression levels combine in an additive manner.

To investigate the involvement of cryptochrome 1 in this UV-B/blue and UV-B/UV-A synergism, the CRY1 null mutant (*hy4-2.23N*) was studied (Fuglevand *et al.*, 1996). The UV-A/UV-B and blue/UV-B light synergistic pathways remained unaffected in the *hy4-2.23N* mutant, implying that the CRY1 photoreceptor is not involved in the synergistic interaction of UV-B light with either UV-A or blue light. Figure 1.7.1 shows the model by Fuglevand *et al.* of the synergistic interaction of the light qualities with *CHS* expression. Further work on the involvement of cryptochromes 1 and 2 in this synergistic interaction was completed by Valentine (1998). The conclusions reached using single and double mutants of *cry1* and *cry2* were that neither the *cry2* single mutant, nor the *cry1cry2* double mutant showed a reduction from that of the

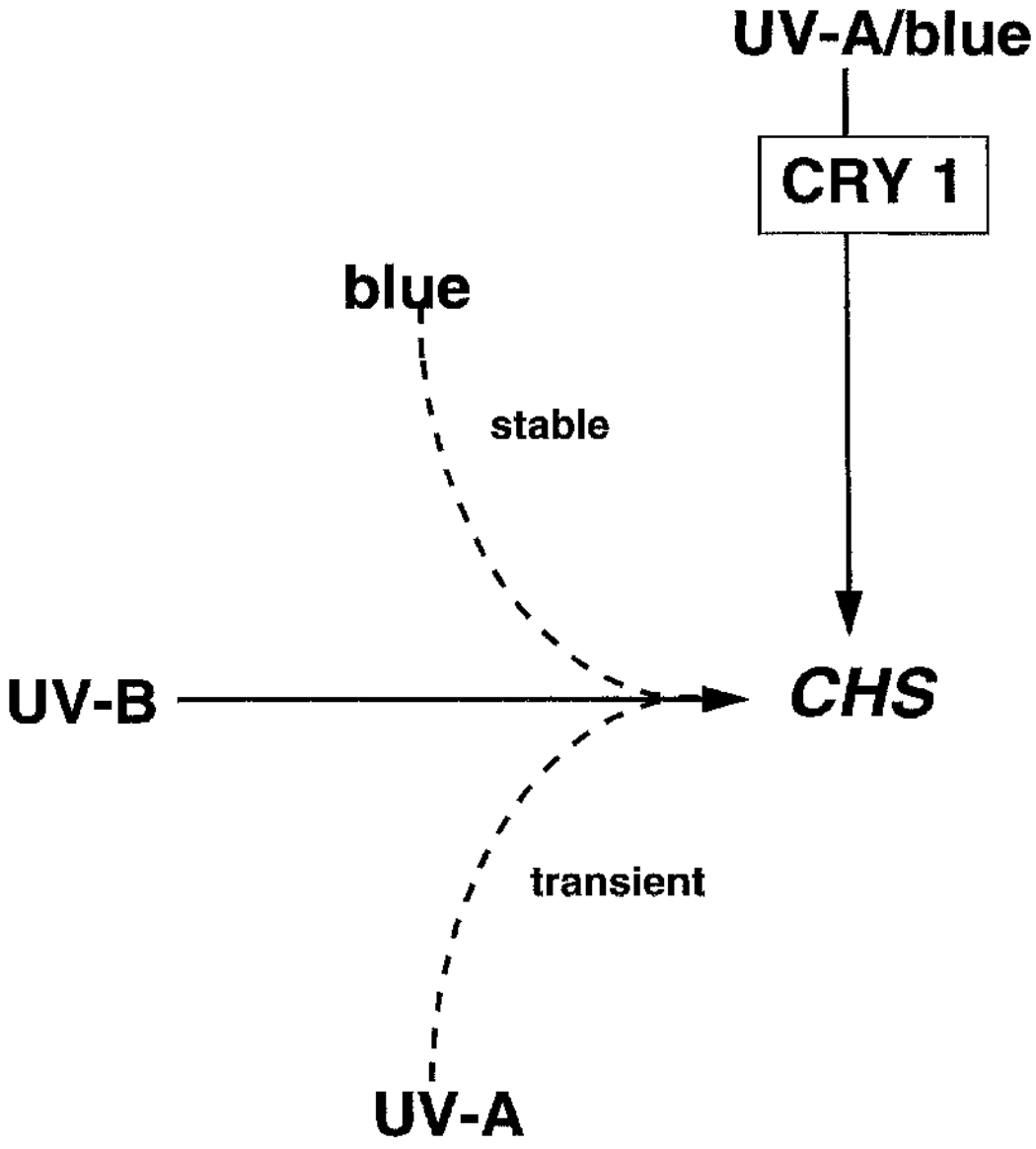


Figure 1.7.1 Synergism Model (Fuglevand *et al.*, 1996)

wild type in *CHS* expression after exposure to UV-B and UV-A/blue light together. This indicates that UV-B/blue synergism is not dependent on either cryptochrome 1 or cryptochrome 2.

1.8 The *icx1* Mutant

Jackson *et al.* (1995) isolated a mutant altered in the light regulation of *CHS* through a transgenic screening approach. After fusing the *SA-CHS1* gene promoter from *Sinapis alba* (white mustard) to the GUS coding sequence, the transgene was introduced into wild type *Arabidopsis thaliana* ecotype Landsberg *erecta*. The gene transfer was accomplished by *Agrobacterium tumefaciens* mediated root transformation. Plants showing kanamycin resistance and easily monitored GUS activity were further investigated. A transformed line, NM4 (non-mutant line 4), homozygous for the *CHS-GUS* transgene was used to produce an isogenic population. The expression of *CHS-GUS* in the NM4 line was shown to be regulated in a similar manner to the endogenous *CHS* gene. Thus, NM4 seeds were mutagenised with ethyl methanesulphonate (EMS) and the M_2 plants were screened for altered GUS expression in both high and low fluence rates of white light.

From the mutant screen, four putative mutants were isolated as having altered levels of GUS activity from that of the NM4 line. Analysis of the endogenous *CHS* expression levels in these four putative mutants removed two from the investigation, where no alteration from that of the NM4 or wild type plants was seen. The other two mutant lines, C10 and A12, showed increased levels of *CHS-GUS* expression and increased levels of endogenous *CHS* transcripts. The *CHS* transcript increase in these two plants was in a non-constitutive manner, that is to say, the level of *CHS-GUS* expression increased with an increasing level of white light. One of these mutants, A12, was further investigated and renamed *icx1* (increased chalcone synthase expression 1).

1.8.1 *icx1* Gene Expression

icx1 has increased chalcone synthase expression in response to white

light. That *icx1* showed non-constitutive expression of *CHS* was further emphasised when the levels of both GUS activity and *CHS* transcript were analysed in both eight day old dark grown seedlings and dark adapted three week old seedlings. The levels of *CHS* transcripts in dark grown and dark adapted *icx1* seedlings were almost negligible, as is the case in similarly treated wild type seedlings.

The effect of white light on *icx1* plants leads to a hyper induction in *CHS* expression and also a two- to threefold increase in anthocyanin accumulation (Jackson *et al.*, 1995). Anthocyanin is one of the downstream products of the flavonoid biosynthetic pathway (Figure 1.5.1) of which *CHS* is a component (see also Section 1.5). In white light it was also found that there was a concomitant increase in transcript levels of *DFR* and *CFI*. This is not surprising as *DFR* and *CFI* act in the flavonoid biosynthetic pathway downstream from *CHS* and leading to anthocyanin accumulation. The increase in anthocyanin accumulation seen in *icx1* plants was not enough to produce a visible colouration of the plant. Other flavonoid products were also increased in the *icx1* mutant. A study completed by M. Hughes in our laboratory on the kaempferol content of *icx1* and wild type plants indicates that there is also an increase in this flavonoid in *icx1* plants. Kaempferol, as can be seen in Figure 1.5.1, is a flavonol and is a product downstream of *CHS* and *CFI*, but not *DFR*. Figure 1.8.1 is a graph of the relative accumulation of kaempferol in *icx1* and wild type plants grown in 20 μmol m^{-2} s^{-1} for two weeks and either continuing treatment at that fluence rate (LW) for 10 days, transferred into high white light (100 μmol m^{-2} s^{-1}) for 10 days (HW) or transferred outside during the day (in Glasgow, summer 1996) and placed in 100 μmol m^{-2} s^{-1} overnight (OUT), also for 10 days. *icx1* has 3.4 fold greater content of kaempferol than the wild type when both are grown OUT, 1.9 fold greater accumulation in LW and 3.0 fold greater accumulation in HW.

The increase in both anthocyanin accumulation and kaempferol content

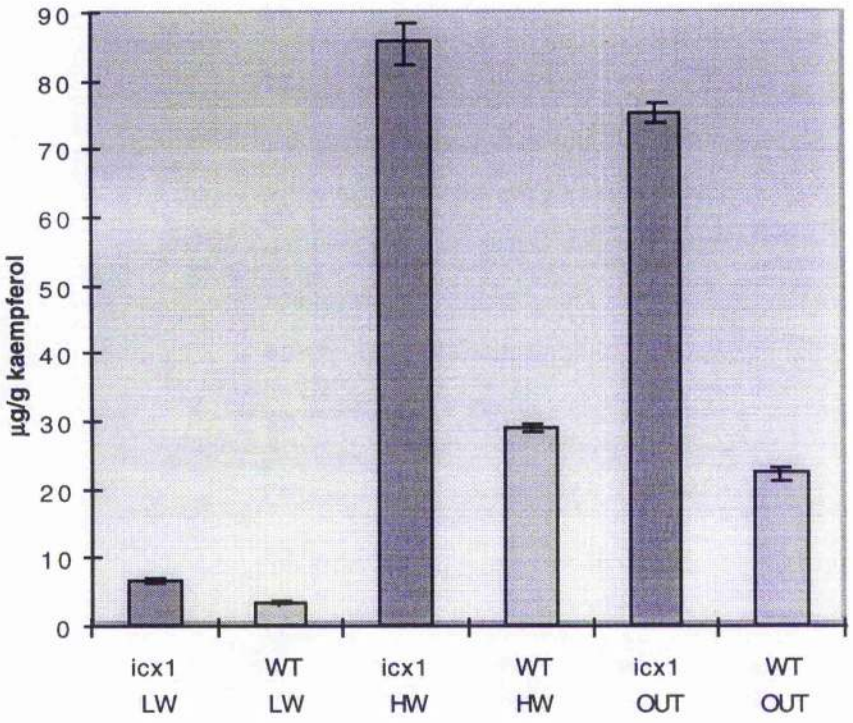


Figure 1.8.1 Kaempferol content in *icx1* and wild type plants
Two week old *Arabidopsis* plants were transferred into high white light (100 µmole m^{-2} s^{-1}) or were placed outside (Glasgow, summer 1996) during daylight hours and placed in high white light overnight for 10 days. Control plants were untreated and remained growing in low white light for the 10 days of the treatment. Plants were harvested and analysed with HPLC (M. Hughes, G.I. Jenkins and A. Crozier unpublished data).

in *icx1*, compared to that of the wild type plants, allows the conclusion to be reached that the *CHS* transcript increases seen are linked to an increase in downstream products.

1.8.2 *icx1* Visible Phenotype

The *icx1* mutation has pleiotropic effects, not only is there increased *CHS* and other flavonoid biosynthetic gene expression, there are phenotypic alterations affecting a number of aspects of plant development (Jackson *et al.*, 1995).

icx1 has narrower leaves than wild type, a trait which is more pronounced when the plants are grown under high white light, though still observable in low white light. The shape of the leaves is not only narrower than the wild type, rather than being rounded or oval shaped the leaves taper to a point and also have a somewhat spiked leaf margin as can be seen in Figure 1.8.2. This exaggerated feature on the leaf margin is thought to be the hydathode, the point where excess water can be secreted from the leaf in times of high water uptake and low transpiration in a process known as guttation.

icx1 has fewer leaf trichomes, with about half the number normally seen on wild type leaves. The seeds of *icx1* are smaller than wild type seeds and they display a slower release of seed mucilage (Jackson *et al.*, 1995). The combination of alterations in *icx1* is similar to that of the *ttg* (*transparent testa glabra*) mutant (Koornneef, 1981) although the alterations themselves are different.

The *ttg* mutant has no trichomes or seed mucilage and an absence of anthocyanin accumulation, which gives the seeds the characteristic transparent testa. The disruption of TTG and ICX1 affects the same aspects of plant development, but in different ways. Crossing *icx1* to *ttg* indicates that *icx1* is non-allelic to *ttg*. Further crosses showed that *icx1* was also non-allelic to a number of trichome mutants: *gl1* (*glabra1*), *gl2* and *gl3* (J. A. Jackson, B. A. Brown and G. I. Jenkins, unpublished).

Jackson *et al.* (1995) suggested that ICX1 acts in the epidermis to

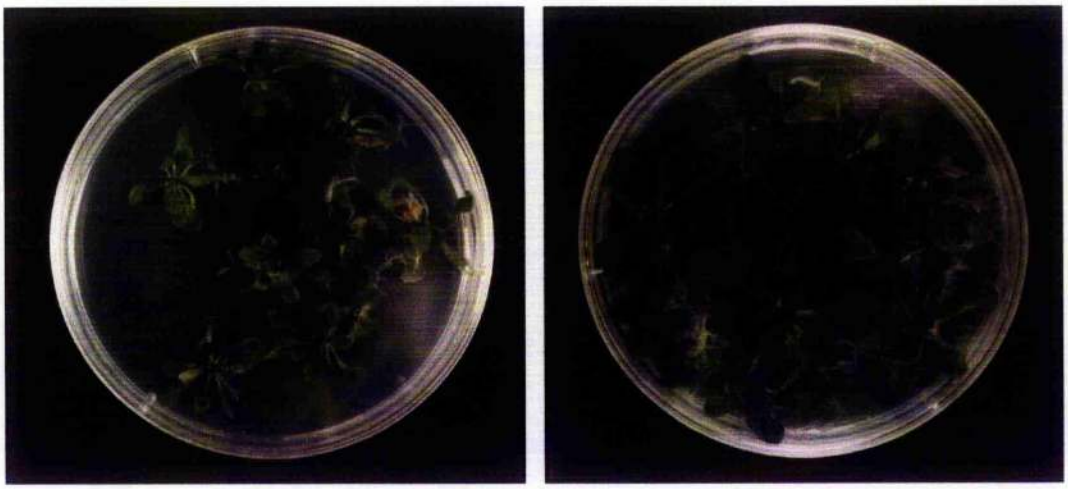


Figure 1.8.2 Visible phenotype of *icx1*
icx1 and wild type plants were grown for 30 days in low white light on solid growth media. Note the exaggerated hydathode and leaf tapering to a point seen in *icx1* as compared to wild type (J. A. Jackson, B. A. Brown and G. I. Jenkins, unpublished data).

regulate gene expression and epidermal development. The mutation could be affecting a negative control mechanism which acts in the light to regulate the amount of *CHS* present in the cell (Jackson *et al.*, 1995).

Other effects of a mutation in ICX1 can be seen with closer study of the epidermal layer. In *icx1* plants the roots are stunted and EM pictures show that the root hair files are disrupted (J. A. Jackson, B. A. Brown and G. I. Jenkins, unpublished). In wild type plants root hairs are only produced along files of epidermal cells, with each root hair file separated from its nearest neighbour by two non-root hair files. In *icx1* the spacing of root hairs is more random. Epidermal alterations have also been seen on *icx1* leaves, where 'sausage shaped' cells are found in the mutant, but not in the wild type. The epidermal alterations in the *icx1* epidermis can be seen via electron microscopy as presented in Figure 1.8.3. It is likely that epidermal cell expansion and/or division is affected.

A study was undertaken of the double mutants of *icx1* with other mutants altered in epidermal aspects, particularly trichome formation. The *icx1 gl1* double mutant lacks trichomes on stems and leaves while the *icx1 gl2* double mutant has fewer trichomes on leaves than either parent, and an intermediate number on stems. The *icx1 gl1* double mutant shows the 'sausage shaped' epidermal cells. The *icx1 ttg* double mutant is similar to *icx1* in leaf shape and size; *ttg* has leaves of similar size and shape to wild type. The lack of anthocyanin in the seeds and leaves, which is characteristic of *ttg*, was seen in the *icx1 ttg* double mutant. The *icx1 ttg* double mutant has no trichomes, like the *ttg* parent, but does display the 'sausage shaped' cells seen in *icx1*. These results indicate that *ttg* is epistatic to *icx1* with regard to trichome formation and anthocyanin biosynthesis. The presence of the *icx1* mutation determines the leaf shape and epidermal cell morphology of the double mutant. *TTG* therefore appears to have little influence on epidermal cell expansion/division.

The pleiotropic phenotype of *icx1* results in alterations in the plant comparable to the effects of a mutation in the *TTG* gene. The phenotype of the

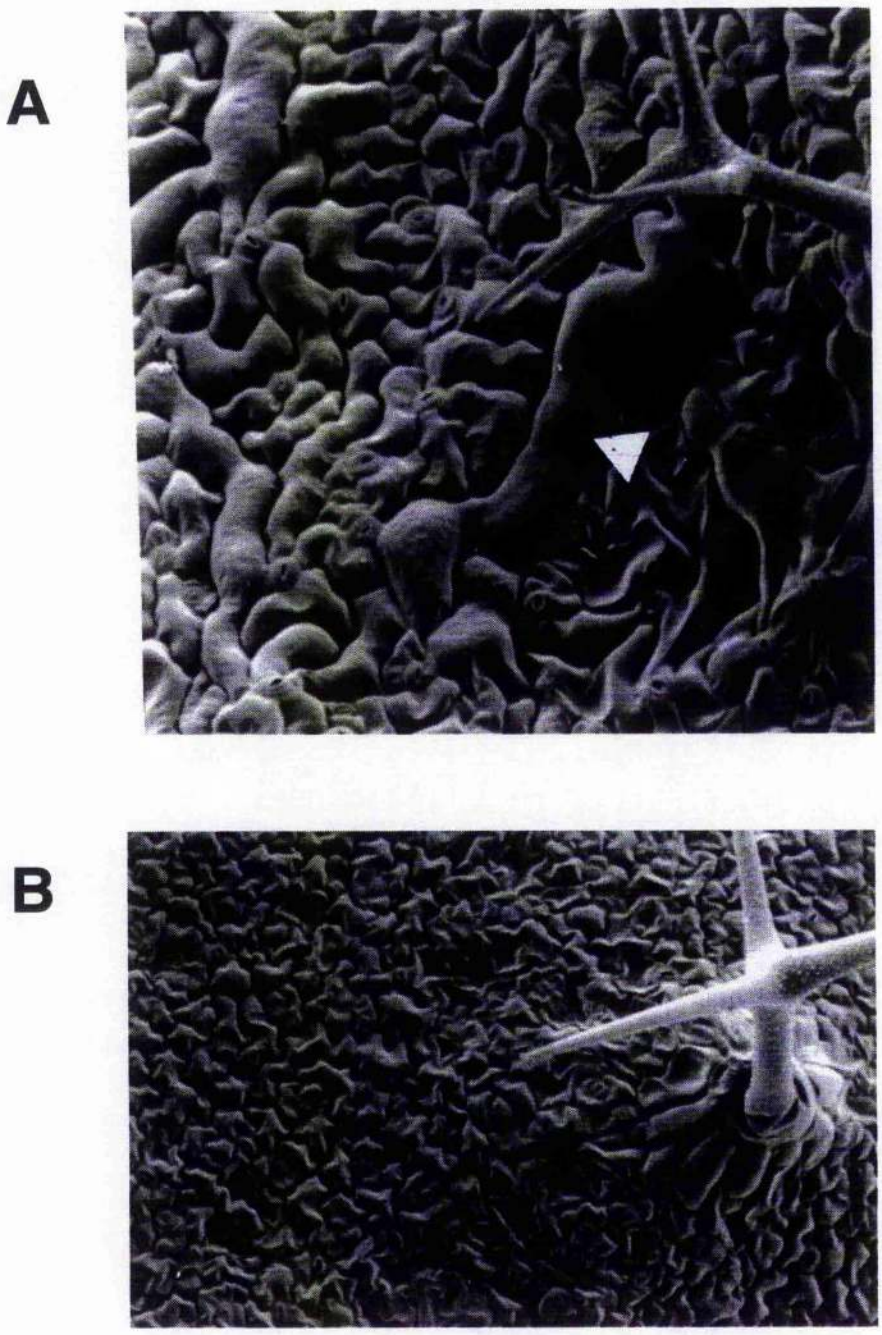


Figure 1.8.3 Phenotypic alteration of epidermal cells in *icx1* mutant plants

Scanning electron micrograph of upper leaf surface showing epidermal cells of icx1 (A) and wild type (B). Note the protruding "sausage shaped" epidermal cells (indicated by the arrow head) which are not seen in the wild type plants. Magnification x 300. (J.A. Jackson, B. A. Brown, G. I. Jenkins, unpublished data)

ttg mutant is rescued by the maize *R* gene (Lloyd *et al.*, 1992) giving rise to the possibility that TTG is or regulates an *R* homologue. Cloning of the *TTG* gene has indicated that it is not an *R* homologue (Schnittger *et al.*, 1998), though the fact remains that *ttg* is rescued by *R* gene expression. The maize R gene encodes a basic-helix-loop-helix protein which can interact with myb proteins C1 or Pl to activate structural genes for anthocyanin biosynthesis (Goff *et al.*, 1992). The *ttg* mutant is altered in a different manner from that of *icx1*, since in *ttg* there is no anthocyanin, whereas *icx1* has increased accumulation. Both mutants are altered in trichome number and seed mucilage biosynthesis (Jackson *et al.*, 1995; Koornneef, 1981). *ttg* has no trichomes and *icx1* has a reduced number. The similarities of function of *TTG* and *ICX1* indicate that they are functioning to regulate similar aspects of development (seed mucilage, trichome number) and regulation (anthocyanin accumulation). The manner of regulation is different, as disruption of the two genes produces different responses.

The tomato (*Lycopersicon esculentum* Mill.) high-pigment-1 (*hp-1*) mutant also shows an increase in anthocyanin accumulation. This is coupled with a short hypocotyl and increased *CAB* and *rbcS* gene expression in the dark (Peters *et al.*, 1998). Similar to *icx1*, the *hp-1* mutants show a light mediated increase in *CHS* expression (Peters *et al.*, 1998; Peters *et al.*, 1989). Unlike *icx1*, *CAB* levels also increase in *hp-1* in inductive light conditions (Peters *et al.*, 1998). In tomato *CHS* and *CAB* are both under phytochrome regulation, which is different to the situation in mature *Arabidopsis* leaves, where *CHS* expression is regulated by blue and UV light (discussed in Section 1.6). It is possible that *ICX1* and *HP-1* are homologues or act in a similar manner, and the differences seen between the phenotypes are a result of the different regulation systems in place in the respective species. That it is difficult to compare mutants in different species has been highlighted recently, as mutations in homologous genes occasionally result in very different phenotypes in different species. A second high pigment mutant *hp-2* has been shown to be a homologue of the *Arabidopsis DET1* gene (Mustilli *et al.*, 1999) but it does not show any de-etiolation responses in the

dark.

icx1 appears to be affecting expression of a gene coding for a lipid transfer protein (LTP) in the epidermis. In *icx1* expression of the *LTP* was increased in red and blue light compared to that of wild type (A. K. Sohal and G. I. Jenkins, unpublished). The alteration of a gene unconnected to flavonoid biosynthesis in *icx1* further supports the possibility that ICX1 acts in the epidermis to regulate gene expression and epidermal development.

Work is continuing to clone the ICX1 gene. ICX1 has been mapped to the lower arm of chromosome 1 between markers m315 and ADH (R. A. Brown, J. A. Jackson, C. E. Johnstone, and G. I. Jenkins, unpublished work).

1.9 Aims of Project

ICX1 acts as a negative regulator of signals leading to *CHS* expression in *Arabidopsis* (Jackson *et al.*, 1995). A major aim of the work presented here was to investigate the role of ICX1 in signal transduction leading to increased *CHS* expression. It was to be ascertained whether ICX1 acts in a particular photoreceptor signal transduction pathway, whether the negative regulation of ICX1 was specific to light signal transduction pathways, and whether the expression of other flavonoid and non-flavonoid biosynthetic genes was also altered in the *icx1* mutant. Genetic analysis of the function of ICX1 was to be investigated via double mutant studies.The role, if any, of ICX1 in UV-A plus UV-B and blue plus UV-B synergism was to be studied.

A further aim of this project was to investigate the interaction between photoreceptor signalling pathways, if there was any, in the regulation of *CHS* expression. Fuglevand *et al.* (1996) reported the synergistic interactions betweenUV-B and both UV-A and blue light, are there more interactions? *CHS* expression in phytochrome A and B mutants can be compared to that of wild type to investigate whether there is a role for PHYA and PHYB in regulation of UV induced *CHS* expression.

Mutant analysis has indicated number of components involved in light

signal transduction (see Section 1.4.2). The use of mutant screens can be tailored to identify as yet unknown components of the light signalling transduction pathways. Isolation of positive regulators of *CHS* expression is possible using appropriate screens. One of the aims of this project was therefore to develop the basis of a screen which would be specific for low *CHS* expressing mutants.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals were obtained from BDH (AnalaR® grade, Poole, Dorset) unless otherwise indicated.

2.1.2 Radiochemicals

[α-^{32}P]dCTP and [α-^{35}S]dATP were supplied by Amersham International (UK).

2.1.3 Plasmids and Bacterial Strains

The plasmids used in this study are described in Table 2.1. *E. coli* XL-1 Blue (Stratagene) and pBluescript KS$^-$ (Promega) were also used for the amplification of plasmids. Additionally, pBI101 (Clontech) was modified and transformed into *Agrobacterium* strain GV3101 (Koncz and Schell, 1986).

2.1.4 Liquid and Solid Bacterial Growth Media

LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride, pH 7.3) was used to grow *E. coli*. In growing *Agrobacterium*, LB or the rich growth medium, YEP (1% (w/v) tryptone, 1% (w/v) yeast extract and 0.5% (w/v) sodium chloride) was used. For plates, 15 g/l agar was added to media prior to autoclaving. Antibiotics were added after sterilisation, when the solution had cooled to 50°C.

2.1.5 Solid Plant Media

Solid media for growth of plants consisted of 1 x Murashage and Skoog (MS) salts, 1 x B5 vitamins, 0.8% (w/v) agar, with or without 2% (w/v) sucrose as appropriate. The medium was sterilised and once the solution had cooled to 50°C it was poured under sterile conditions into 90 mm petri dishes and allowed to set. Solid medium for selection of transformed seed consisted of 1/2

Probe	Insert	RE	Plasmid
CAB	1.65 kb	*Eco*RI	pAB165
CFI	0.72 kb	*Eco*RI/*Sal*I	pBluescript KS+
CHS	0.7 kb	*Hin*dIII	pUC19
DFR	1.15 kb	*Eco*RI/*Sal*I	pBluescript KS+
PAL	4.0 kb	*Eco*RI/*Hin*dIII	pUC19
rbcS	1.9 kb	*Eco*RI	pUC9
α–TUB	1.3 kb	*Hin*dIII/*Bgl*II	pcf4-2

Table 2.1 Plasmids used in this study

The plasmids used for amplification of DNA inserts are described, the size of the inserts used for hybridisation analysis is indicated (Insert). The restriction enzymes required to digest the plasmids to obtain the inserts is also indicated (RE). All plasmids contain ampicillin resistance. References for the inserts: CAB (Leutwiler *et al.*, 1986); CFI and DFR (from Shirley, as described in Jackson *et al.*, 1995); CHS and PAL (Trezzini *et al.*, 1993); rbcS (obtained from M. Timko); α-TUB (Silflow *et al.*, 1985).

MS salts, 1 x B5 vitamins, 0.8% (w/v) agar. The medium was sterilised and once the solution had cooled to 50°C, the appropriate antibiotic was added prior to swirl mixing and pouring into 90 mm petri dishes.

Solid media for selection of plants resistant to 5-FC consisted of 1/2 MS salts, 1 mg/ml 5-FC, 2.5 mM MES, 100 mM sucrose, 0.8% (w/v) agar (pH 5.7). The MS salts, sucrose and agar were added to water and autoclaved, 5-FC was dissolved in MES, filter sterilised and added to the rest of the medium after cooling. The medium was swirl mixed and poured into 90 mm petri dishes.

2.1.6 Antibiotics

Antibiotics were supplied by Sigma Chemical Company (UK). All antibiotics used were dissolved in water and filter sterilised. Ampicillin and kanamycin stocks were at 50 mg/ml and used at a final concentration of 50 µg/ml. Gentamycin stock solution at 25 mg/ml was used at a final concentration of 25 µg/ml.

2.1.7 DNA Modifying Enzymes

All restriction enzymes were obtained from Life Technologies (Paisley, UK) together with their reaction buffers, which were provided at a 10 x concentration.

2.1.8 Soil

The soil and sand used to grow plants was obtained from William Sinclair Horticulture Ltd (Lincoln, UK). 1 part Silvaperl® sand was mixed with 4 parts soil and autoclaved for an hour.

2.2 General Laboratory Procedures

2.2.1 pH measurement

The pH of solutions other than phenol was measured using a Jenway pH meter 3320. The pH of solutions containing phenol was measured using Whatman Narrow Range pH paper (pH 6.0-8.0) (Whatman, Maidstone, UK).

2.2.2 Autoclaving

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

2.2.3 Filter Sterilisation

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

2.2.4 Solutions and Equipment for RNA Work

Solutions used in RNA preparation were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC, Sigma), left to stand overnight then autoclaved. All glassware used in the preparation of RNA was baked at 180°C for four hours. Sterilised plasticware was used throughout.

2.3 Plant Material

2.3.1 Seed Stocks

Arabidopsis thaliana cv Landsberg *erecta* seeds were obtained from the Arabidopsis Stock Centre (Nottingham, UK). The *icx1* mutant plants were isolated in this background after EMS mutagenesis, see Jackson *et al.* (1995). In all data presented here the original *icx1* line backcrossed twice to wild type (*Arabidopsis thaliana* cv Landsberg *erecta*) was used. The phytochrome A mutant, *phyA* (*fhy2-1* allele, Whitelam *et al.*, 1993) was provided by N. P. Harberd (John Innes Centre, Norwich) The phytochrome B mutant *phyB*, (*hy3-1 allele*), the downstream light signalling mutant *hy5* (*hy5-1* allelle), and the cryptochrome 1 mutant used in this study (*hy4-2.23N* allele) (Koornneef *et al.*, 1980), were all obtained from the Arabidopsis Stock Centre (Nottingham, UK). The phytochrome A, phytochrome B null double mutant in the Landsberg *erecta* background, *phyA-1phyB-1* was kindly provided by Prof. Garry Whitelam (University of Leicester, UK). JH10, the *CHS* promoter-*GUS* coding transgene containing line in L. *erecta* background was produced in collaboration with Bernd Weisshaar's laboratory (MPI, Köln, Germany); John Hays made the *CHS*-

GUS cassette, the plants were transformed by Ulrike Hartmann and the *GUS* expression of the line was tested by Geeta Fuglevand.

2.3.2 Growth and Harvesting of Soil Grown Plants

Arabidopsis seeds were sown in pots on damp, sterile soil. The pots were covered with cling film and vernalised by cold treatment (4°C) for 2-3 days to break seed dormancy. Plants were grown for three weeks under low fluence rate white light (20 μmol m^{-2} s^{-1}) at 20°C prior to treatment. After treatment leaf tissue was frozen directly in liquid nitrogen and, if stored, was kept at -80°C.

2.3.3 Surface Sterilisation of Seeds

Seeds which were to be sown on sterile growth media or selection plates were surface sterilised.

2.3.3.1 Large Scale Seed Surface Sterilisation

The seeds were placed in a magenta jar in filter paper packets then immersed for 2 minutes in 70% (v/v) ethanol. After draining off the ethanol, the seeds were then immersed in a bleach solution (10% (v/v) sodium hypochlorite and 0.01% (v/v) 1000 x Triton) for 10 minutes with occasional shaking. The bleach was poured off the seeds and the seeds were then washed 5 times in sterile dH_2O under sterile conditions. The filter paper packets containing the seeds were left to dry overnight in a laminar flow hood.

2.3.3.2 Small Scale Seed Surface Sterilisation

When less than (approximately) fifty seeds per seed type were to be sterilised, the following method was used. Seeds were placed in a 1.5 ml Eppendorf® and 0.5 ml of 70% (v/v) ethanol was added. After 2 minutes the 70% ethanol was removed with a pipette and 0.5 ml of the bleach solution described in Section 2.3.3.1 was added. The Eppendorf® was then closed and inverted, ensuring all seed remained in the solution. Ten minutes after the addition of the sodium hypochlorite solution, the bleach solution was removed by pipette and the seeds washed with sterile distilled water which was then

pipetted out. The washing step was repeated four more times. Once the final wash was completed as much water as practical was removed from the Eppendorf®. The seeds were then resuspended in 400 µl sterile 0.1% (w/v) agar solution. The seeds were then imbibed at 4°C for 3-4 days prior to sowing on a solid media plate.

2.3.4 Growth of Seedlings in Darkness

Seeds were surface sterilised as described in Section 2.3.3 prior to sowing under sterile conditions onto plates of growth medium as described in 2.1.5. Plates were wrapped in tin foil and placed for 2-4 days at 4°C in the dark. Seeds were then exposed to 1 hour of 20 µmol m^{-2} s^{-1} white light at room temperature to ensure germination, before being placed in a dark growth room. Seedlings were treated on the fourth day after removal from cold. RNA extraction was done either directly on fresh tissue or on tissue that had been frozen in liquid nitrogen immediately after the end of the treatment. Sugar assays were made on fresh tissue, which had been weighed and washed with copious dH_2O to remove any sugars remaining on the surface of the seedlings.

2.3.5 Plant Crosses

Plants were grown for 4 to 5 weeks in 20 µmol m^{-2} s^{-1} white light until flower bolts formed. After paring down three or four buds to the stigma, and removing any other buds, pollen from the other parent plant was placed on the stigma. Both parent plants were used for pollination of the other parent plant. The artificially pollinated buds were isolated from other flowering bolts and harvested after the siliques had begun to dry on the plant. Plants were screened for phenotype in the F2 generation.

2.4 Illumination of Plant Material

Illuminations were carried out in controlled environment rooms at 20°C. Control plants were maintained in the appropriate growth conditions for the

duration of the light treatments and harvested at the same time as the last sample.

2.4.1 Light Sources

The spectra of all the light qualities were measured with a spectroradiometer (Macam SR9910) and are presented in Figure 2.4.1. White light was provided by warm white fluorescent tubes (Osram, Munich, Germany). UV-A/blue light was provided by Sylvania 40W T12 blue fluorescent tubes (GTE Sylvania, Shipley, UK). Blue light was obtained by covering the above tubes with "Moonlight Blue" filter (No. 183, Lee Filters, England) to cut out wavelengths below 380 nm. UV-A light was produced by Sylvania F35W/B1-2B blacklight-blue fluorescent tubes, emitting wavelengths between 320 and 390 nm. UV-B light was obtained by covering UVB-313 ultra violet fluorescent tubes (Q-Panel Co., USA) with cellulose acetate (Diacel 120 microns FLM 400110, Film Sales) to omit UV-C, which was changed after every 24 hours of use. Red light was obtained by covering high output white fluorescent tubes (Phillips, PL-L 55W/83/4P) with "Deep Golden Amber" filter (No. 135, Lee Filters). Far-red light was provided by Toshiba FL20S FR-74 tubes. The required fluence rate was achieved by varying the number of tubes used in the growth area and adjusting the distance between the plants and the light source. The fluence rates of the light regimes are described for each experiment. Where light spectra indicate output outwith the wavelengths specified, it was ascertained that the light was insufficient to induce *CHS* expression.

2.4.2 Fluence Rate Measurement

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

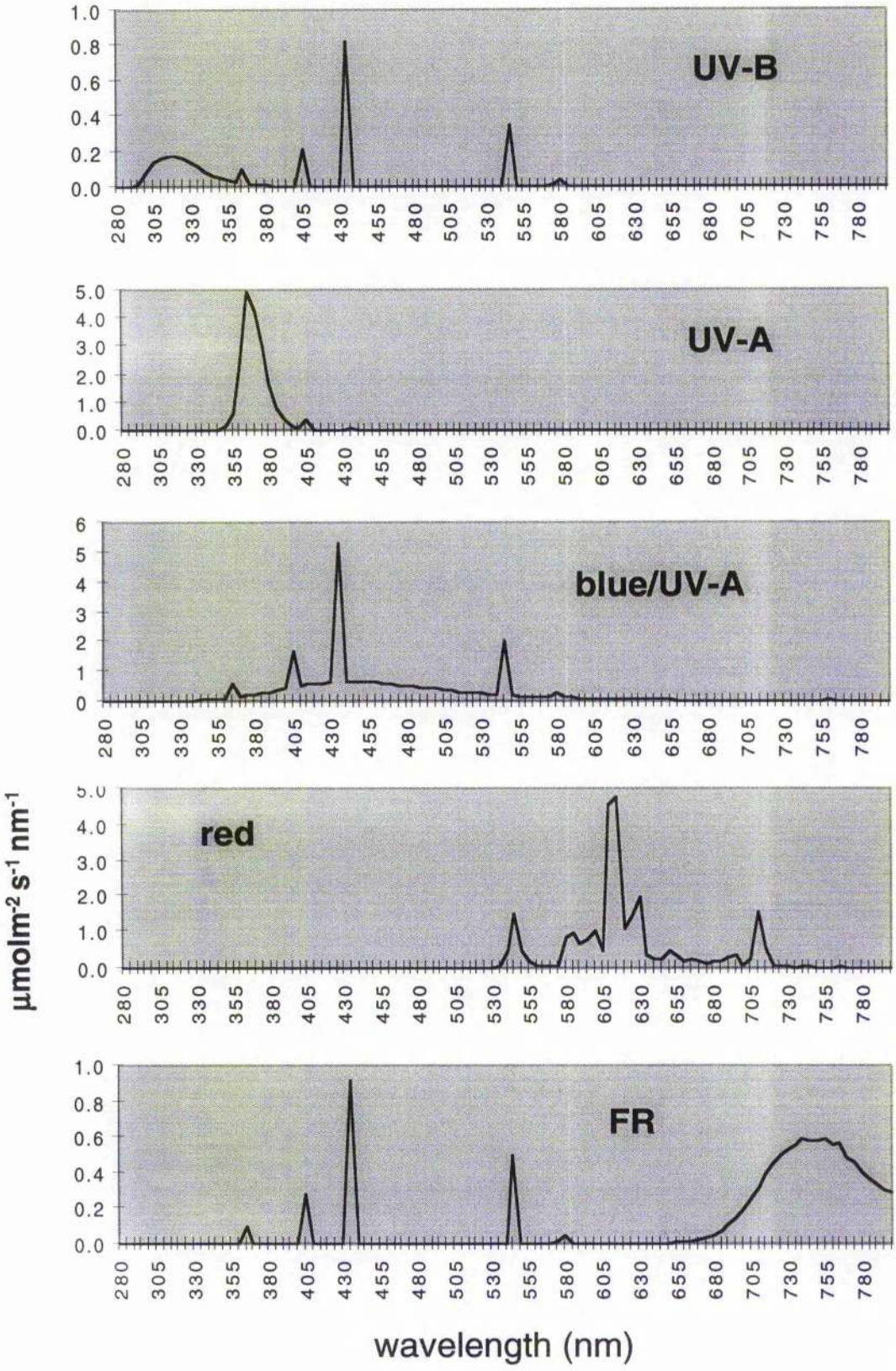


Figure 2.4.1 Spectra of the different light qualities used throughout the study

The spectral photon distribution of each of the light qualities used, measured using a spectroradiometer (Macam SR9910) in $\mu molm^{-2}s^{-1}$ nm^{-1}. Note that the x axes differ betweeen graphs.

2.5 Isolation of Total RNA from Plant Material

2.5.1 Preparation of Phenol

Five hundred grams of phenol (detached crystals) was melted at 65°C for 30 minutes and 0.58 g of 8-hydroxyquinoline added before the phenol had cooled. The warm solution was saturated with 100 mM Tris-HCl (pH 8.0) and mixed vigorously for 10 minutes. After allowing the solution to settle, the upper phase was removed by aspiration and the pH of the phenol measured. Aspiration and washing with 100 mM Tris-HCl (pH 8.0) was repeated until the pH of the phenol was greater than 7.6. The upper phase was then replaced with 100 ml of 10 mM Tris-HCl (pH 7.6) and the solution stored at 4°C.

2.5.2 RNA Extraction Following Jackson *et al.* (1995)

RNA was either extracted using a modified version of the Flowgen Purescript RNA kit, as described in Section 2.5.3, or following the method of Jackson *et al.* (1995).

Approximately 0.5 g of frozen plant tissue was ground into a fine powder in liquid nitrogen using a mortar and pestle. The powder was then transferred to a 15 ml Corex® tube containing 4 ml extraction buffer (5 M guanidine thiocyanate (Sigma), 25 mM tri-sodium citrate, 2 mM EDTA (di-sodium salt), 50 mM Tris-HCl pH 7.6, 0.5% (w/v) sarkosyl (Sigma), 1 mM ß-mercaptoethanol) and briefly vortexed. Equal volumes (4 ml) of phenol and chloroform were added and the solution vortexed once more before spinning at 3,000 *g* for 10 minutes at 4°C. After centrifugation, the upper layer was removed to a clean tube, 2 volumes of ice cold *iso*-propanol were added and the tube inverted several times before being transferred to -80°C for 15 minutes.

After precipitation, RNA was pelleted by centrifugation at 12,100 *g* for 10 minutes at 4°C and the supernatant discarded. The pellet was washed with 1 ml 70 % (v/v) ethanol then spun once more for 10 minutes. The pellet was air dried and resuspended in an appropriate volume of DEPC-treated dH_2O.

The purity of the preparation was estimated by measuring the OD at

260 nm and 280 nm. A pure preparation was taken as having a 260/280 nm ratio of 1.8–2.0. RNA preparations were stored at -20°C or -80°C.

2.5.3 Flowgen Kit RNA Extraction Method

The Flowgen Purescript RNA isolation kit (Flowgen, Staffordshire, UK) was also used to extract RNA. The Flowgen kit instructions were followed, with the exception of an additional chloroform extraction to increase the purity of the resultant RNA sample.

0.05-0.3 g of frozen plant tissue was ground to a fine powder in liquid nitrogen with a mortar and pestle. The powder was then transferred into a sterile 1.5 ml Eppendorf® tube containing 0.3 ml of Flowgen Cell Lysis Solution (containing citric acid, EDTA, SDS) and vortexed for 10 s. 100 µl of Flowgen Protein-DNA precipitation solution (containing citric acid, NaCl) was added to the solution and the Eppendorf® inverted several times to mix the solution. After placing the solution on ice for 5 minutes the sample was spun for 5 minutes at 14,000 rpm in a microcentrifuge. The supernatant was added to 0.5 ml chloroform, mixed, and microcentrifuged for 20 minutes at 14,000 rpm. The aqueous layer was then transferred to a fresh Eppendorf® containing 0.3 ml *iso*-propanol and mixed by inversion of the tube. The RNA was pelleted by microcentrifugation for 3 minutes and the supernatant discarded. The pellet was washed with 0.3 ml 70 % (v/v) ethanol (made with DEPC treated dH_2O). The pellet was air dried and was then resuspended in 12 to 25 µl DEPC treated dH_2O.

The purity of the preparation was again estimated as described in Section 2.5.2., and the RNA preparations were stored at -20°C or -80°C.

2.6 Amplification and Preparation of Plasmid DNA

2.6.1 Preparation of Competent *E. coli* Cells

DH5α cells were streaked onto a minimal plate (LB only, no antibiotics) and placed in an incubation oven at 37°C overnight. A single colony was picked

from the plate and used to inoculate a 5 ml LB culture which was placed in a 37°C shaking incubator overnight. This culture was then added to a further 100 ml of LB and the culture grown until the absorbance at 550 nm was 0.48. The culture was then chilled on ice for 5 minutes, before transferring into two sterile falcon tubes and spinning at 1000 *g* for 10 minutes at 4°C. The supernatant was removed, and the pelleted cells were resuspended in 20 ml of 1 M KAc, 1 M $RbCl_2$, 1 M $CaCl_2$, 1 M $MnCl_2$, 80% (v/v) glycerol pH 5.8., care was taken in resuspending the cells. After chilling on ice for a further 5 minutes the spin step was repeated and after removal of the supernatant, each pellet was carefully resuspended in 2 ml 100 mM MOPS, 1 M $CaCl_2$, 1 M $RbCl_2$, 80% (v/v) glycerol, pH 6.5. The cells were chilled on ice for 15 min. After separation into 0.1 ml aliquots in 1.5 ml Eppendorf® tubes the cells were frozen in liquid nitrogen and stored at -80°C until use.

2.6.2 Transformation of Competent *E.coli* Cells

Frozen competent cells prepared as described in 2.6.1 were thawed on ice. Approximately 1-10 ng of plasmid DNA, in a volume of 5-10 µl or 6 µl ligation solution was added to the Eppendorf® and the tube gently shaken before being returned to ice for 30 minutes. After this period, the cells were heat shocked at 42-44°C for 90 s and returned to ice for 5 minutes. LB medium (0.5 ml) was added to the cell suspension and the Eppendorf® shaken at 37°C for 60 minutes 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 cells resuspended in 0.1 ml LB medium. The cell suspension was plated on agar with the appropriate antibiotic. As a control, competent cells were treated as above, in the absence of DNA, and plated on antibiotic-containing plates.

2.6.3 Preparation of Competent *Agrobacterium* Cells

A single colony of *Agrobacterium* was grown up overnight at 28°C in a 5 ml YEP medium. 2 ml of this culture was added to 50 ml of YEP in a 250 ml

flask, which was shaken vigorously at 28°C until the culture had an OD_{600} of between 0.5 and 1.0. The culture was chilled on ice prior to centrifugation at 3,840 *g* for 5 minutes at 4°C. The supernatant was discarded and the pellets resuspended in 1 ml of ice cold 20 mM $CaCl_2$. The *Agrobacterium* cells were divided into 0.1 ml aliquots in prechilled Eppendorfs® and frozen in liquid nitrogen. The transformation competent cells were then stored at -80°C until use.

2.6.4 Transformation of Competent *Agrobacterium* cells

1 µg of plasmid DNA was added to the frozen cells. The *Agrobacterium* and plasmid were placed in a 37°C water bath for 5 minutes. 1 ml of YEP was added to each tube then the cells were incubated at 28°C for 2 to 4 hours, with gentle shaking, to allow the cells to express antibiotic resistance. The cells were pelleted for 30 seconds at 10,000 *g* and the supernatant was discarded, the pellet was resuspended in 0.1 ml YEP and spread on a YEP agar plate containing the appropriate antibiotic. The plates were incubated at 28°C and colonies appeared after 2 to 3 days. As a control, cells were treated for transformation in the absence of additional DNA.

Single cell colonies of the putatively transformed cells were used to inoculate 3 ml YEP overnight cultures containing the appropriate antibiotics. Minipreps were carried out on these overnight cultures and the plasmid digested with restriction enzymes to check that the plasmids contained the DNA insert.

2.6.5 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 LB medium, supplemented with the appropriate antibiotic, in a 30 ml Universal tube. The culture was grown overnight with constant shaking at 37°C (in the case of *E.coli*) or 28°C (for *Agrobacterium*). 1 ml of the overnight culture was transferred

to a 1.5 ml Eppendorf® tube and centrifuged at 10,000 *g* for 5 minutes. The supernatant was discarded and the bacterial pellet resuspended by vortexing in 0.2 ml resuspension solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 µg/ml RNAse A). Once fully resuspended, 0.2 ml of cell lysis solution (0.2 M NaOH, 1% (w/v) SDS) was added and the solution mixed by gentle inversion. Next, 0.2 ml neutralisation solution (1.32 M potassium acetate) was added and the solution mixed as before. The tube was centrifuged at 10,000 *g* for 5 minutes. Meanwhile, a minicolumn was placed on a vacuum manifold (Promega) and the barrel of a syringe attached to it, 1 ml of Wizard™ Minipreps DNA purification resin was aliquotted into the syringe barrel. The supernatant was then added to the syringe barrel and a vacuum applied to pull the slurry into the minicolumn. After breaking the vacuum, 2 ml of wash solution (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 2.5 mM EDTA, 55% (v/v) ethanol) was added to the syringe barrel and the vacuum reapplied. Once the wash solution had passed through the minicolumn, the vacuum was left on for a further 30 s to dry the minicolumn. The vacuum was broken and the minicolumn removed from the syringe barrel and transferred to a 1.5 ml Eppendorf® tube. This was microcentrifuged for 20 s to further dry the resin. The minicolumn was then transferred to a clean 1.5 ml Eppendorf® tube, 50 µl of dH_2O added, and then left at room temperature for 1 minute. Plasmid DNA was eluted by microcentrifugation for 20 s. The minicolumn was then discarded.

2.7 Quantification of DNA, RNA and Oligonucleotides

An aliquot of the nucleic acid solution to be quantified (usually 1 µl of RNA, and 2-5 µl of DNA or oligonucleotide) was diluted to an appropriate volume (100 µl for RNA and 1 ml in the cases of DNA or oligonucleotides) with sterile dH_2O. The absorbance of the solution was measured between 250 nm and 350 nm against a sterile dH_2O blank. Absorption values were taken at 260, 280 and 300 nm. Estimation of the quality or quantification calculations were

made using A_{260} - A_{300} and A_{280} - A_{300} values for each sample. In this way any contaminating carbohydrate present in the sample did not affect quantification. An absorbance at 260 nm (minus the absorbance at 300 nm) of 1 was taken to indicate the following concentrations:

Form of nucleic acid	Concentration (µg/ml)
Double Stranded DNA	50
Single stranded DNA and RNA	40
oligonucleotide	~20

2.8 Agarose Gel Electrophoresis

All agarose (*ultra* PURE™) was supplied by Life Technologies, Paisley. Electrophoresis of DNA was carried out following one of two methods. DNA which was not used for further manipulation was electrophoresed as described in Section 2.8.1. If DNA was to be enzymatically modified after purification, the method described in Section 2.8.2 was used.

2.8.1 Electrophoresis of DNA

The appropriate concentration of agarose (0.6-2.0% (w/v)) was added to the appropriate volume of 1 x TBE (0.1 M Tris-Borate, 2 mM EDTA, pH 8.3) required for the gel being cast. The agarose suspension was heated in a microwave until the agarose had completely dissolved. The solution was allowed to cool to around 60°C, at which point ethidium bromide was added to a final concentration of 1 mg/ml and the gel solution poured into the electrophoresis apparatus. After allowing the gel to set for 30 minutes, enough 1 x TBE running buffer was added to just submerge the gel. DNA samples to be loaded were mixed with 1/10 volume of loading buffer (20% (v/v) glycerol, 0.025 M NaOH, 0.25% (v/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF) and loaded with a pipette. Electrophoresis was carried out at 15-80 mA until the bromophenol blue had migrated two-thirds of the way down the gel. The gel was then visualised under UV light (Spectroline® transilluminator, Model

TC-312A).

2.8.2 Electrophoresis of DNA to be used for Cloning

When DNA was run on a gel for separation of restriction fragments which were to be used for ligations or further manipulation, it was electrophoresed in the absence of borate ions. The gels were made as described in Section 2.8.1 except for the substitution of 1 x TBE buffer with that of 1 x TAE (2 M Tris-HCl, 5.71 % (v/v) acetic acid, 0.05 M EDTA, pH 8.0) buffer.

2.8.3 Denaturing Electrophoresis of RNA

This method was used for gels that were to be blotted for northern hybridisation analysis. 1.3 g of agarose was added to 72 ml of dH_2O. The agarose suspension was then heated in a microwave until the agarose had dissolved. Once the agarose solution had cooled to 60°C, 10 ml of 10 x MOPS (0.2 M MOPS, 50 mM NaOAc, 10 mM EDTA pH 7.0) and 18 ml of formaldehyde (37% (v/v), Sigma) were added and the gel mixed by swirling prior to pouring into the electrophoresis apparatus. After 30 minutes the gel had set and was transferred to 4°C for a further 30 minutes. The gel was then placed in the gel tank and submerged in 1 x MOPS.

For a given gel, an equal amount (5-10 µg) of RNA was added to 14.5 µl denaturing buffer (1 µl 10 x MOPS, 3.5 µl formaldehyde, 10 µl formamide). The samples were made up to an equal volume with DEPC-treated distilled water. The samples were incubated at 65°C for 5 minutes to denature the RNA. After addition of 4 µl of loading buffer (50% (v/v) glycerol, 1 mM EDTA, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol FF) to each sample, the samples were spun down and loaded into the gel wells with a pipette. Electrophoresis was carried out at 25-90 V until the bromophenol blue had migrated two thirds of the way down the gel. The gel was then visualised under UV light (Spectroline® transilluminator, Model TC-312A).

2.9 Isolation of DNA Fragments from Agarose Gels

2.9.1 Phenol/Chloroform Extraction of DNA

The DNA fragment of interest was initially separated from residual DNA by agarose gel electrophoresis as described in Section 2.8.2. The DNA of interest was identified by size compared to a DNA size marker and visualised under UV light by ethidium bromide staining. The fragment was then excised with a clean scalpel blade, cut into small pieces and separated (0.1 g) into 1.5 ml Eppendorf® tubes. 0.5 ml of phenol was added to each Eppendorf® and the mixture vortexed until the gel fragments had emulsified. Each tube was then frozen in liquid nitrogen and immediately centrifuged at 10,000 *g* for 10 minutes. The upper phase was removed to a fresh Eppendorf® tube and an equal volume of phenol and chloroform added. After vortexing, the solution was centrifuged at 10,000 *g* for 2 minutes and the upper layer removed to a clean tube. An equal volume of chloroform was added and the 10,000 *g*, 2 minute, centrifugation step repeated. The upper layer was removed to a fresh tube and 1/10 volume 3 M sodium acetate (pH 5.5) added along with 2 volumes of ethanol. DNA was precipitated at -20°C for at least one hour and then centrifuged for 5 minutes at 10,000 *g*. After decanting the supernatant, the DNA pellet was washed with 70% (v/v) ethanol before centrifuging once more. The supernatant was removed, the pellet was air dried for 10 minutes and then resuspended in an appropriate volume of dH_2O. This procedure yielded about 50–60% recovery of the DNA.

2.10 DNA Manipulation

2.10.1 Digest of DNA with Restriction Endonucleases

DNA to be digested was prepared in a solution of 1 x the appropriate buffer and 1-20 units of the restriction enzyme(s) added to a maximum of 10% (v/v) of the final volume. Reactions were incubated at the appropriate temperature for 2-24 h and monitored, where sufficient DNA was available, by

running an aliquot of the reaction on an agarose gel against uncut plasmid DNA and marker DNA (1 kb ladder, Life Technologies and lambda DNA digested with *Eco*RI and *Hin*dIII, Promega).

2.10.2 DNA Ligation

After restriction endonuclease digestion and gel purification of DNA, the ligation reaction was as follows. The amount of insert added to a solution of T4 ligase buffer was calculated by the equation:

(vector (ng) x kb size of insert)/(kb size of vector) x molar ratio of (insert/vector)

amount of insert (ng) to use

0.1-1 units of T4 ligase were then added. The final volume of the solution gave a concentration of 1 x T4 ligase buffer and the T4 ligase did not exceed 10% (v/v) of the final volume. The reaction was incubated at room temperature for 2 h before adding to competent cells for transformation as described in Section 2.6. After spreading on LB plates containing the appropriate antibiotic and incubating overnight at 37°C, putative positives were picked and used to inoculate 3 ml overnight cultures. The plasmids were then checked by restriction digestion and gel electrophoresis, measuring the resultant fragments against a 1 kb ladder (Life Technologies). Control reactions were as above, in the absence of the smaller DNA fragment.

2.10.3 Klenow 5' Overhang Fill-in

If a blunt end is required on DNA cut by a restriction enzyme which leaves a cohesive (sticky) end, the Klenow fragment can be used. In order to fill-in a cohesive end where the 3′ strand is longer than the 5′ end the following procedure was used. 10 µg of DNA was digested and phenol extracted to remove the restriction enzyme. After ethanol precipitation the pellet was resuspended in 35 µl of dH_2O and 5 µl of Klenow 10 x buffer added. 10 µl of 10/4 dNTPs (made up of equal volumes of 10 mM dATP, dTTP, dGTP and

dCTP) and 2-3 units of Klenow were then added to the DNA. The mixture was left to incubate at room temperature for an hour, then heat treated at 65°C for 5 minutes. The DNA was precipitated by phenol/chloroform extraction and the pellet resuspended in 20 µl of dH_2O. The DNA could then be further manipulated.

2.11 Northern Blotting

RNA was separated on a denaturing agarose gel as described in 2.8.3. A wick of Whatman 3 MM paper was placed onto a support and soaked in 20 x SSC (3 M NaCl, 0.3 mM *tri*-sodium citrate). The wick was also dipped into a reservoir of 20 x SSC. The gel was placed on top of the wick with the well side of the gel face down. Air bubbles were removed from between the wick and the gel. A piece of nylon membrane (Hybond-N, Amersham) was cut to marginally larger than the gel, and after soaking in water for 5 minutes, placed on top of the gel, ensuring that there were no air bubbles between the gel and the nylon filter. An additional piece of filter paper, cut to the same size as the nylon filter, was soaked in 20 x SSC and placed on top of the nylon. That part of the wick which was not covered by the gel was screened with cling film, to prevent direct absorption of 20 x SSC by the dry tissues which were then placed on top of the filter paper. A glass plate weighted with 500 g was placed on the top of the tissues. The blot was left overnight, then disassembled. The gel and filter were examined under UV light to assess the transfer of RNA from the gel to the nylon. After successful transfer, the filter was briefly rinsed in sterile dH_2O and air dried at room temperature. RNA was crosslinked to the nylon membrane by UV treatment (UVP, CL-1000, ultraviolet crosslinker, 12,000 Joules m^{-2} s^{-1}).

2.12 Radiolabelling of DNA

2.12.1 Preparation of DNA to be Labelled

DNA sequences to be labelled were isolated from plasmids by restriction enzyme digestion and gel purification as described in 2.9. The

amount of the DNA was estimated by running an aliquot of the fragment on an agarose gel as described in Section 2.8.1 and comparing it to known amounts of DNA loaded onto the same gel.

2.12.2 Random Priming of dsDNA

DNA was labelled with [α-^{32}P]dCTP using the Rediprime DNA labelling kit supplied by Amersham International in accordance with the manufacturer's instructions. Approximately 25-50 ng of DNA, prepared as described in 2.12.1, was diluted to a volume of 45 µl with sterile dH_2O in a 1.5 ml screw top Eppendorf® tube. The DNA was denatured by heating the sample to 95°C in a boiling water bath for 5 minutes and then briefly microcentrifuged. The denatured DNA was added to the labelling mix (dATP, dGTP, dTTP, exonuclease-free 'Klenow' enzyme and random nonamer primers) and the mixture reconstituted by gently flicking the tube until the blue colour of the labelling mix was evenly distributed. After a brief microcentrifugation, 50 µCi (3,000 Ci mmol^{-1}) of [α-^{32}P]dCTP was added to the reaction mixture and the tube incubated at 37°C for 20 minutes. After this incubation, the reaction was stopped by the addition of 5 µl of 0.5 M EDTA. Unincorporated radionucleotides were removed using a sephadex mini column as described in Section 2.12.3. For use in hybridisation analysis, DNA was denatured by heating to 95-100°C for 5 minutes. The sample was chilled on ice until required.

2.12.3 Separation of Labelled DNA from Unincorporated Radionucleotides Using Spin Columns

To remove any unincorporated radionucleotides, the probe was spun through a dry Sephadex mini column. A hole was made in the base of a sterile 0.5 ml Eppendorf® with a 23 or 25 gauge needle. 10 µl of sterile glass beads (Jencons Ballotini No.11, Jencons (Scientific) Ltd, Leighton Buzzard, UK) in dH_2O were pipetted into the Eppendorf® to plug the hole. The Eppendorf® was then filled with Sephadex TE previously made by autoclaving Sephadex G50 medium (Pharmacia, Milton-Keynes, UK) in a 20 fold volume of TE (10

mM Tris-HCl HCl, 1 mM EDTA, pH 8.0). The 0.5 ml Eppendorf® was placed inside a 1.5 ml Eppendorf® with a 12 gauge needle hole in the base. Both Eppendorfs® were suspended at the top of a 10 ml test tube. The column was then spun at 1,000 *g*, for 4 minutes. The spin was repeated after additional Sephadex TE was added to the 0.5 ml Eppendorf®.

The minicolumn in the 0.5 ml Eppendorf® was transferred to a fresh 1.5 ml screw top Eppendorf® and the solution of radioactively labelled DNA and unincorporated radionucleotides pipetted onto the top of the minicolumn. The minicolumn with sample was spun at 1,000 *g*, for 4 minutes. The incorporated radioactivity was collected in the 1.5 ml Eppendorf® and the unincorporated radioactivity remained in the minicolumn, which was discarded.

2.13 Hybridisation Analysis of Northern Blots

Investigation of transcript levels by northern analysis was carried out. The DNA that was radiolabelled and used to hybridise to northern filters is described in Table 2.1. An α-tubulin (TUB) probe was used as a loading control. This was the 1.3 kb *Hin*dIII/*Bgl*II cDNA insert from pcf4-2 encoding *Chlamydomonas reinhardtii* α-tubulin (Silflow *et al.*, 1985). All comparisons of blots in any one figure section are representative data from a single filter.

2.13.1 Hybridisation of Nylon Filters

northern blots were analysed using the method of Church and Gilbert (1984). Nylon filters, prepared as described in 2.11, were placed in Techne (Cambridge, UK) glass bottles and 20 ml of prehybridisation solution (0.5 M phosphate buffer pH 7.2, 7% (w/v) SDS, 10 mg/ml BSA) added. The bottles were placed in Techne hybridisation ovens and the nylon filters prehybridised for at least 2 h at 55°C. The radioactive denatured probe was added to the prehybridisation solution and the hybridisation was left to proceed overnight.

2.13.2 Washing of Northern Blots

After hybridisation, filters were washed twice for 10 minutes in an

appropriate volume of 2 x SSC, 1% (w/v) SDS at 55°C. If background radioactivity of the filters remained high, they were then washed at increased stringency (1 x SSC, 1% (w/v) SDS) at 55°C. Stringency could then be increased with increasing temperature of the wash solution.

2.13.3 Autoradiography

Filters to be autoradiographed were sealed in heat sealable plastic, and exposed to Fuji X-Ray film (type RX) in a film cassette with intensifying screens at -80°C for the appropriate length of time.

2.13.4 Stripping Filters of Bound Radiolabelled Probes and Blocking Agents

After hybridisation and autoradiography, bound probe and blocking agents were removed by washing the filter in boiling 0.1% (w/v) SDS. The solution was allowed to cool to room temperature. The boiling wash was repeated until no radioactivity could be detected by a Geiger counter. The nylon filters were sealed in plastic and autoradiographed overnight to verify that the radiolabelled probe had been removed.

2.13.5 Re-use of Radiolabelled DNA

After hybridisation, probes in prehybridisation solution were poured off into falcon tubes and stored at 4°C. To re-use, the whole hybridisation mix was heated to 95-100°C for five minutes, allowed to cool to 55°C at room temperature, then added to previously prehybridised filters. After overnight hybridisation, filters were treated as described in 2.13.2 and 2.13.3.

2.14 Polyacrylamide Sequencing of DNA

2.14.1 dsDNA Sequencing

Sequencing reactions were made using the Sequenase Version 2.0 DNA Sequencing Kit (Amersham Life Science, Illinois, USA). Oligonucleotide primers specific for the sequence were obtained from Cruachem Ltd. (Glasgow,

UK) and diluted to 0.5-1 pmol μl^{-1}.

Plasmid DNA which was to be sequenced was amplified and purified as described in 2.6, and additionally treated with 100 µg/ml RNase A for one hour at 37°C. The RNase was removed by phenol/chloroform extraction and an aliquot of DNA quantified by running on a 1 x TBE gel with lambda DNA of known concentration. In a 0.5 ml Eppendorf®, 3-5 µg of plasmid were denatured by adding 0.1 volumes of 2 M NaOH, 2 mM EDTA and incubating for 30 minutes at 37°C. 0.1 volumes of 2 M sodium acetate (pH 4.5-5.5) was added to neutralise the mixture and the DNA precipitated for 15 minutes at -80°C after addition of 2-4 volumes of ethanol. The DNA was pelleted and washed with 70% (v/v) ethanol. The DNA pellet was resuspended in 7 µl of dH_2O before the addition of 2 µl of Sequenase buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) and 1 µl of primer (0.5-1.0 pmol). The primer was annealed to the DNA by heating the template-primer mix to 65°C for 2 minutes and allowing the mix to cool slowly to below 35°C. The template-primer was then kept on ice. The following solutions were then added to the mix; 1 µl 0.1 M DTT, 2 µl Labelling mix (1.5 µM dGTP, 1.5 µM dCTP, 1.5 µM dTTP), 0.5 µl [α-^{35}S]-dATP and 2 µl Sequenase Version 2.0 T7 DNA polymerase (13 units μl^{-1}, in 20 mM KPO_4, pH 7.4, 1 mM DTT, 0.1 mM EDTA, 50% glycerol, diluted 8 fold in Enzyme Dilution buffer (10 mM Tris-HCl, pH 7.4, 5 mM DTT, 0.5 mg ml^{-1} BSA)). The labelling reaction was then incubated for 2-5 minutes at room temperature prior to the termination reaction.

The labelling reaction was terminated by adding 3.5 µl of the labelling reaction to each of four tubes G, A, T and C, containing 2.5 µl of prewarmed ddGTP, ddATP, ddTTP, ddCTP termination mixes, respectively, at 37°C. The termination reactions contain 80 µM dGTP, 80 µM dATP, 80 µM dTTP, 80 µM dCTP, 50 mM NaCl and 8 µM ddNTP, specific for each termination mix. After adding the labelling reaction to the termination reaction, the incubation of the G, A, T, C tubes was continued for a further 3-5 minutes at 37 °C. Reactions were stopped by the addition of 4 µl Stop Solution (95% (v/v) formamide, 20

mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF) and were stored on ice until loading onto the sequencing gel.

2.14.2 Electrophoresis of Polyacrylamide Sequencing Gels

The sequencing gel was made using a SequaGel®-6 (National Diagnostics, Hull, UK) system. 0.8 ml of 10% (w/v) ammonium persulphate was added to polymerise 100 ml of SequaGel®-6 buffer-monomer mix (5.7% (w/v) acrylamide, 0.3% (w/v) methylene bisacrylamide, and urea in 0.1 M Tris-borate, 2 mM EDTA, pH 8.3 (1 x TBE)). The solution was briefly swirl mixed, then poured into the gel cast, taking care to avoid trapping air bubbles. The gel was allowed to polymerise for at least 2 hours. The gel was then placed in the sequencing gel tank, 1 x TBE added as running buffer, and the gel preheated by running at 2500 V for at least an hour before the first sample was loaded.

After running at 2500 V for 2-4 h the sequencing gel was dismantled and dried onto filter paper. The gels were then exposed to X-ray film at room temperature for up to a week.

2.15 *Agrobacterium* Mediated Vacuum Infiltration

The *Agrobacterium* mediated vacuum infiltration method described by Andrew Bent (Staskawicz Lab, UC-Berkeley) on the Arabidopsis net (www.bio.net/hypermail/ARABIDOPSIS/9312/0083.html) was used and is described below. A glycerol stock of *Agrobacterium* containing pBICHSCD was used to streak YEP plates containing 50 µg/ml kanamycin (Kan) and 25 µg/ml gentamycin (Gn) and incubated for 2 days at 28°C. Single colonies from that plate were used to set up 5 ml YEP (50 µg/ml Kan and 25 µg/ml Gn) overnight cultures which were grown at 28°C, shaking at greater than 200 rpm. A flask containing 500 ml of YEP (Kan and Gn as above) was inoculated with two of the 5 ml overnight cultures and was left to incubate for at least 24 hours at 28°C, shaking at greater than 200 rpm.

The following day, once the culture had grown to a concentration of greater than A_{600}=2.0 the *Agrobacterium* was spun down at 3,840 *g* for ten

minutes and the pellet was resuspended in three times the culture volume of infiltration media (1/2 x MS salts, 1 x B5 salts, 5% (w/v) sucrose and benzylaminopurine (in DMSO) made up fresh) giving an approximate absorbance of A_{600}=0.8.

Plants had been grown for four weeks under 20 µmol m^{-2} s^{-1} white light, to flower bolting stage. Flower bolts were cut back four days prior to vacuum infiltration, to allow more buds to form on secondary flowering bolts. Siliques which had formed and flowers which had already opened were removed from the plants, to reduce the number of untransformed seed in the final harvest. The plants were inverted and immersed in the *Agrobacterium* infiltration medium inside a vacuum jar and a vacuum applied for 3 minutes. The vacuum was released quickly and plants removed from the infiltration medium. The pots of plants were laid on their side inside a plastic bag to maintain high humidity and returned to 20 µmol m^{-2} s^{-1} white light in growth chambers. The following day, the plants were removed from the bags, placed upright in 100 µmol m^{-2} s^{-1} white light and left to set seed. Seed was harvested, surface sterilised and plated out on selection plates (1/2 x MS salt, 50 mg/ml Kan, 0.8% (w/v) agar), vernalised and left to grow for a week under 20 µmol m^{-2} s^{-1} white light.

Plants which did not lose their colour were transplanted onto soil and grown up for seed.

2.16 GUS Expression and Visualisation

2.16.1 GUS Staining

To ascertain whether plants were expressing the ß-glucuronidase gene (*GUS*) individual leaves were incubated with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). A stock solution of X-Gluc was made by dissolving X-Gluc in DMSO and adding distilled water to obtain a final concentration of 25

mg/ml. The leaves were incubated in 1 mg/ml X-Gluc, in GUS extraction buffer (50 mM NaPO4, pH 7, 10 mM EDTA, 0.1% (v/v), Triton X 1000, 0.1% (w/v) sarkosyl, 10 mM ß-mercaptoethanol) at 37 °C overnight. The presence of an active *GUS* gene was indicated by the characteristic blue staining of the leaf tissue after this treatment. GUS converts X-Gluc into a water insoluble blue precipitate, dichloro-dibromo-indigo.

2.16.2 Histological Sections

Plant samples for histological staining were fixed in 0.3 M D-manitol, 10 mM MES, 0.3% (v/v) formaldehyde, pH 5.6, for 45 minutes and incubated with X-Gluc in GUS extraction buffer as described in 2.16.1. After freezing in liquid nitrogen the samples were fixed in Aquamount and sectioned on a microtome. The 10 and 20 µm sections were placed on slides and could be observed under a microscope.

2.17 Anthocyanin Extraction

100 mg of plant tissue was harvested into a 1.5 ml Eppendorf®, the tissue ground in 1% (v/v) HCl in methanol and left to extract overnight, shaking at 4°C. The following day a chloroform extraction of the plant tissue was made and 400 µl of the aqueous supernatant was removed, added to 600 µl of dH_2O and quantified.

Anthocyanin was quantified by absorbance measurement on an absorbance spectrophotometer at A_{657} and A_{530}. Anthocyanin units were calculated as $A_{530} - A_{657}$. Anthocyanin was calculated as anthocyanin units per gram of tissue.

2.18 Sugar Assay

2.18.1 Total Sugar Extraction and Assay

10-100 mg of tissue was harvested, weighed, and washed with distilled water. The tissue was placed in a 1.5 ml Eppendorf® with 0.5 ml of 80% (v/v) ethanol and the sugars extracted by incubating the tissue at 70°C for 1 h. The tissue was pelleted for 1 minute at 10,000 *g* and the supernatant transferred into a fresh 1.5 ml Eppendorf®. The samples were then dried in a speedy-vac before resuspending the sugars in 100 µl of resuspension buffer (100 mM imidazole, pH 6.9, 5 mM $MgCl_2$). 5 µl of the sugar extract was added to a microtitre well containing 195 µl of reaction cocktail. The reaction cocktail consisted of 200 µl of 100 mM ATP and 200 µl of 200 mM NADP were added to 19.1 ml of resuspension buffer and gluconate-6-phosphate dehydrogenase (G6PDH) added to a final concentration of 0.4 units per 195 µl. The extract and reaction cocktail were then incubated for 10 minutes at 37°C. The OD of the sample at 340 nm was taken as A1. 0.3 units of hexokinase was then added to each microtitre well, the samples mixed and incubated for a further 10 minutes at 37°C. This value at A_{340} was designated A2. 0.3 units of phosphoglucoisomerase (PGI) was added to each sample and after incubation as above, the value of A3 measured at A_{340}. The final absorbance value of A4 was calculated by measuring the OD at 340 nm after addition of 0.8 units of invertase to each well and a 10 minute incubation at 37°C.

2.18.2 Calculation of total sugar content in seedlings

Total sugar content of seedlings was calculated using the following method:-

A2-A1 gives the $\Delta A_{glucose}$, A3-A2 gives the $\Delta A_{fructose}$, and (A4-A3)/2 gives the $\Delta A_{sucrose}$.

The calculation for the concentration of each sugar is as follows

$c = (V \times MW)/(e \times d \times v \times 1000) \times \Delta A$ [g/l],

where

c = concentration

V = final volume (ml)

v = sample volume (ml)

MW = molecular weight of substance to be assayed (g mol^{-1})

d = light path (cm)

e = absorption coefficient of NADPH at 340 = 6.3

The resultant values for each sugar were pooled and the total sugar content was calculated as mg sugar per gram fresh weight of seedlings.

2.19 Statistical Analysis

Standard error was calculated and is shown on all bar graphs.

Chapter 3: Characterisation of the *Arabidopsis icx1* Mutant

3.1 Introduction

Jackson *et al.* (1995) described the use of a chimaeric gene introduced into *Arabidopsis* plants to isolate mutants altered in *CHS* gene expression in response to light. An isogenic line of transgenic plants expressing the *GUS* gene driven by the white mustard (*Sinapis alba*) *CHS* promoter was mutagenised by EMS and screened for altered light induction of the transgene. Two mutants with increased *CHS* expression in response to light were isolated. These mutants showed light induction of *CHS* expression, and not constitutive expression. This indicated that they were mutants altered in control of light regulation of *CHS* expression.

One of the mutants isolated, *icx1* (for increased chalcone synthase expression 1) was chosen for further study. The *icx1* mutation has a pleiotropic phenotype which was retained after back crossing the mutant plant twice to wild type (Jackson *et al.*, 1995, J. A. Jackson, R. A. Brown and G. I. Jenkins, unpublished). *icx1* has an altered leaf morphology, which is more pronounced when the plants are grown under higher fluence rates of light (100 μmol m^{-2} s^{-1}). *icx1* has leaves which are narrower than those of the wild type and unlike the round wild type leaves, taper to a point. *icx1* leaves have fewer trichomes than the wild type. 'Sausage shaped' cells can be seen on the surface of the *icx1* leaves, which are not found in the wild type. The *icx1* mutant also has slower release of seed mucilage, which was detected by ruthenium red staining, and the seeds are smaller than those of the wild type. The root morphology of *icx1* is also somewhat altered from that of the wild type, in *icx1* plants, the roots are much shorter and have disorganised production of root hairs.

The visible phenotype displayed by *icx1* indicates that the mutation in the *ICX1* gene has affected both the expression of light regulated genes and epidermal development. The range of effects of the mutation are similar to

those of the *ttg* mutant (Koornneef, 1981), but *icx1* is clearly different from *ttg*. Previously presented work on the *icx1* mutant is described in more detail in Section 1.8.

The work detailed in this section is a characterisation of the role of ICX1 in wild type *Arabidopsis*, as determined by comparison with a mutant deficient in the ICX1 gene product. What are the effects of disrupting ICX1? The questions raised and answered are as follows:

- Does ICX1 act downstream of a particular photoreceptor in mature leaves?
- Does ICX1 function throughout development?
- Does ICX1 act downstream of phytochrome in dark grown seedlings?
- Is the increase in light mediated *CHS* transcript expression due to an increase in the amount of cryptochrome-1 present in *icx1*?
- Has the mutation in *ICX1* affected the expression of other light regulated genes?
- Is ICX1 involved in the spatial expression of *CHS*?
- Does ICX1 only act downstream of light mediated signals?
- Where does ICX1 act with respect to other known light signal transduction proteins?
- Is ICX1 involved in the Blue/UV-B or UV-A/UV-B synergistic responses?

3.2 *CHS* Expression in Plants Treated with Red, Far Red, Blue, UV-A and UV-B Light

icx1 plants have increased *CHS* expression compared to that of wild type in white light (Jackson *et al.*, 1995). It can be investigated whether ICX1 acts downstream of a particular photoreceptor to engender this increase in white light, or if it functions downstream of more than one photoreceptor. Comparison of the *CHS* expression in wild type and *icx1* plants in specific light qualities will provide an answer to this question. Before comparing *icx1* to wild

type, it must first be clear how wild type plants respond to these light qualities.

3.2.1 *CHS* Expression in Wild Type Plants

The *CHS* induction of wild type plants in the experimental set up used is described here. In this investigation plants were grown up under non-inductive conditions, that is, at 20 μmol m^{-2} s^{-1} of warm white light (referred to as "low white light" throughout). This light level was chosen as at this fluence rate there is no observable induction of *CHS* transcript expression, and therefore any increase in *CHS* transcript expression can be easily monitored. Three weeks after germination the plants were exposed to higher fluence rates of various light qualities and subjected to northern analysis.

The light qualities used were red (600-700 nm), far red (700-800 nm), blue (390-500 nm) UV-A (320-390 nm) and UV-B (280-320 nm). Treatments described as blue/UV-A are from blue fluorescent tubes emitting blue light with up to 15% UV-A light present. In treatments described as blue light, blue tubes were covered with a UV-A absorbing filter as detailed in Section 2.4.1. As described in the following section, *CHS* transcript accumulation increased in wild type plants after exposure to blue, blue/UV-A, UV-A or UV-B light.

In 110 μmol m^{-2} s^{-1} blue light, after 12 hours of blue light illumination an increase in *CHS* expression in wild type plants can be seen (Figure 3.2.1). Wild type plants treated with 82 μmol m^{-2} s^{-1} blue/UV-A light (containing 7 μmol m^{-2} s^{-1} of UV-A light) show an increase in *CHS* expression after 2 hours, this trend continues with a peak at 6-8 hours after initial illumination (Figure 3.2.2). In UV-A light alone, as seen in Figure 3.2.3, *CHS* expression is also increased; as with the blue/UV-A light an increase in *CHS* expression is visible after 2 hours. *CHS* expression in UV-A light again peaks after 6-8 hours of exposure after which the level of accumulation begins to decrease.

Figure 3.2.4 shows the induction of *CHS* transcript levels in 3.3 μmol m^{-}

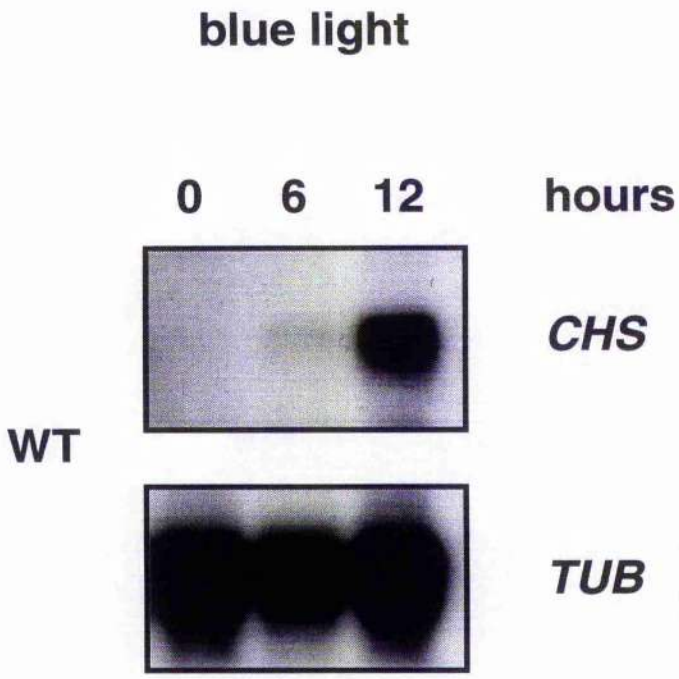


Figure 3.2.1 *CHS* expression in wild type plants in response to blue light

Three week old low white light grown plants were exposed to 110 µmol m^{-2} s^{-1} blue light. Plants were harvested after the times indicated and total RNA isolated.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

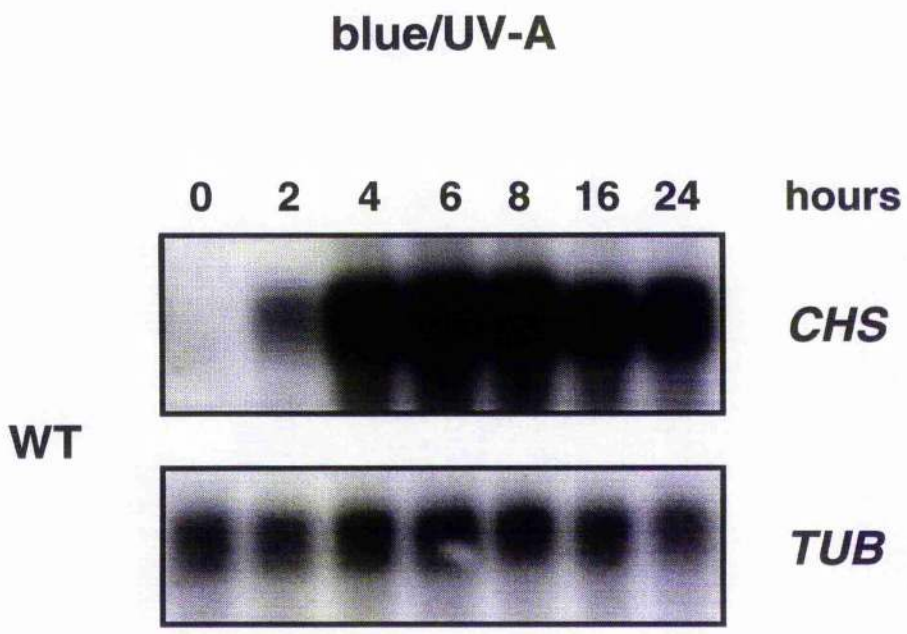


Figure 3.2.2 *CHS* expression in wild type plants after blue/ UV-A light exposure

Three week old wild type plants were exposed to 82 µmol m^{-2} s^{-1} blue light (containing 7 µmol m^{-2} s^{-1} UV-A) prior to harvesting. The 0 hour sample indicates the untreated control. Total RNA was extracted from plant leaves. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

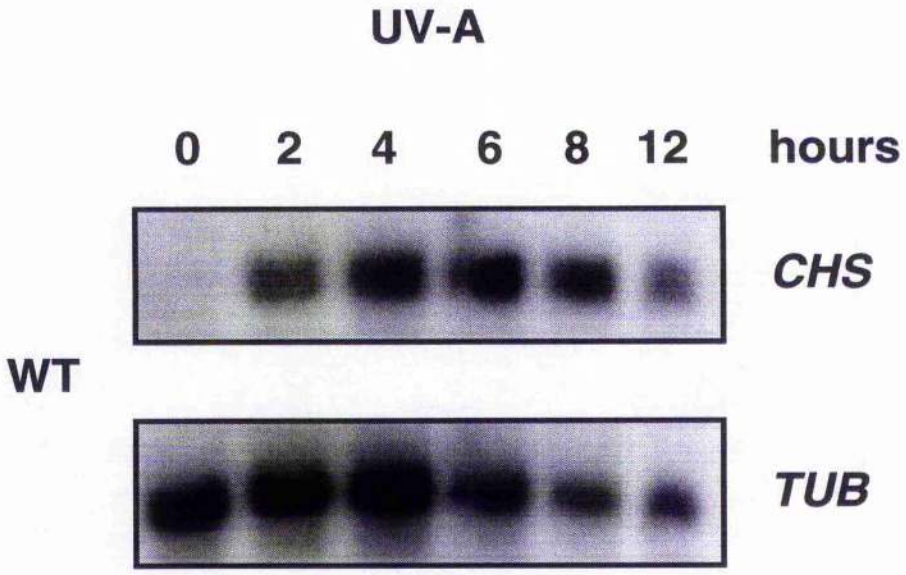


Figure 3.2.3 *CHS* expression in wild type plants after UV-A light exposure

Three week old plants grown in low white light were transferred into 100 μmol $m^{-2} s^{-1}$ UV-A light. Leaves were harvested after the number of hours noted and total RNA was extracted. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

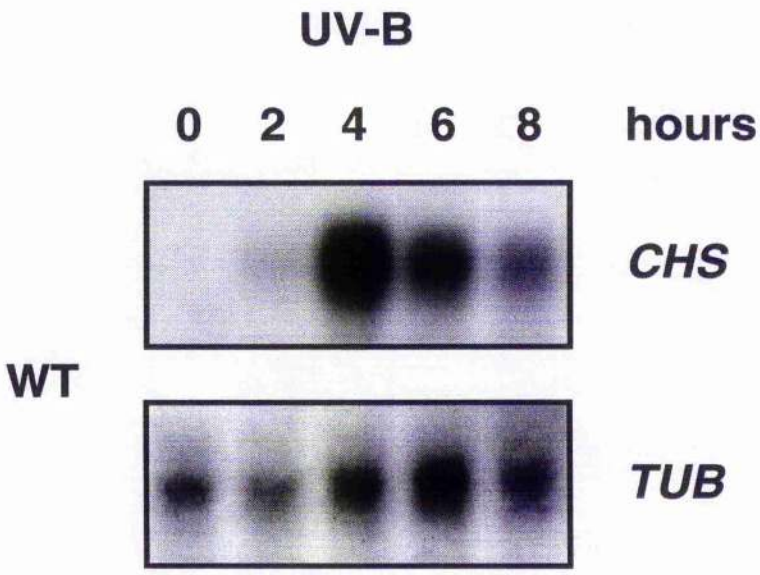


Figure 3.2.4 *CHS* expression in wild type plants after UV-B light exposure

Three week old, low white light grown WT plants treated for the noted times at 3.3 µmol m^{-2} s^{-1} of UV-B light. Leaf tissue was harvested and total RNA extracted.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

^{2} s^{-1} UV-B light. *CHS* expression reaches a peak after four hours of UV-B illumination which then decreases somewhat by six hours and continues to decrease, as can be seen after 8 hours of UV-B exposure. In blue/UV-A the kinetics of induction of *CHS* are different from those in UV-B. As seen by comparison of Figure 3.2.2 with Figure 3.2.4, blue/UV-A and UV-B light results in a similar initial rate of transcript accumulation, in UV-B light the *CHS* accumulation is more transient. In blue/UV-A light, a plateau of transcript accumulation is reached after six hours of exposure to 80 µmol m^{-2} s^{-1} and the expression level is still high after 24 hours. Whereas with UV-B light, *CHS* transcript accumulation has peaked and is declining before the maximal induction in blue/UV-A or UV-A light is seen. This indicates that there are at least two separate light inductive pathways leading to increased *CHS* transcript accumulation, one from UV-B which initiates a more transient *CHS* transcript accumulation than that of the inductive pathway from blue/UV-A light. These results are consistent with those presented by Fuglevand *et al.* (1996).

The induction of *CHS* transcripts in blue/UV-A is different both kinetically and in magnitude to the level of induction in UV-B light. Figure 3.2.5 shows a comparison of the induction of *CHS* over time after blue, UV-A or UV-B light. The *CHS* expression seen at the peaks of induction by UV-A and UV-B light are of a different magnitude under the conditions mentioned. Figure 3.2.5 shows the level of induction of *CHS* after four hours of UV-B light and after six hours of UV-A light. It can be seen that the level of *CHS* induction in UV-B light is not as great as that in response to UV-A light.

When three week old wild type plants were exposed to either red or far red light, no induction of *CHS* transcripts above that of the low white control was seen. The *CHS* expression levels of plants exposed to red, far red or UV-A light are shown in Figure 3.2.6. It can be seen that when plants are exposed to 6 hours of red light at 65 µmol m^{-2} s^{-1} or far red light at 55 µmol m^{-2} s^{-1} no increase in the expression of *CHS* above that of the low white control can be

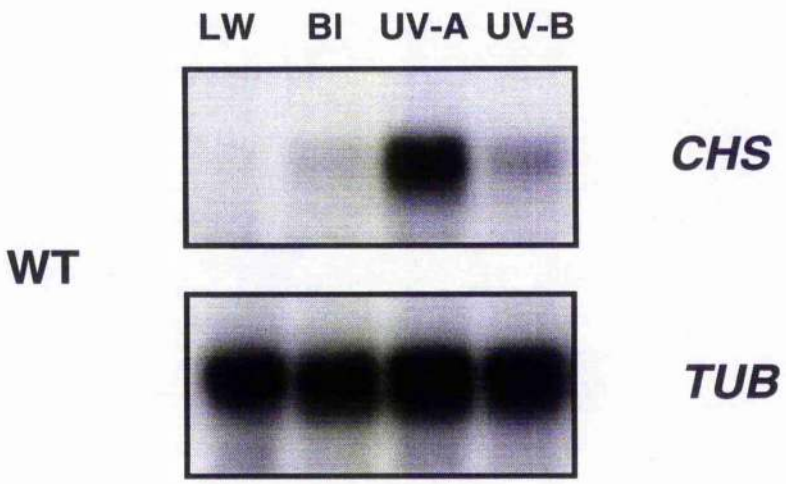


Figure 3.2.5 Comparative level of *CHS* expression in wild type plants exposed to blue, UV-A and UV-B light.

Three week old plants were transferred into either 6 hours of Blue light at 80 µmol m^{-2} s^{-1}, (Bl), 6 hours of UV-A light at 80 µmol m^{-2} s^{-1}, (UV-A) or 4 hours of UV-B light at 3.3 µmol m^{-2} s^{-1} (UV-B). LW is the untreated control.

Total RNA was extracted from the leaves. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

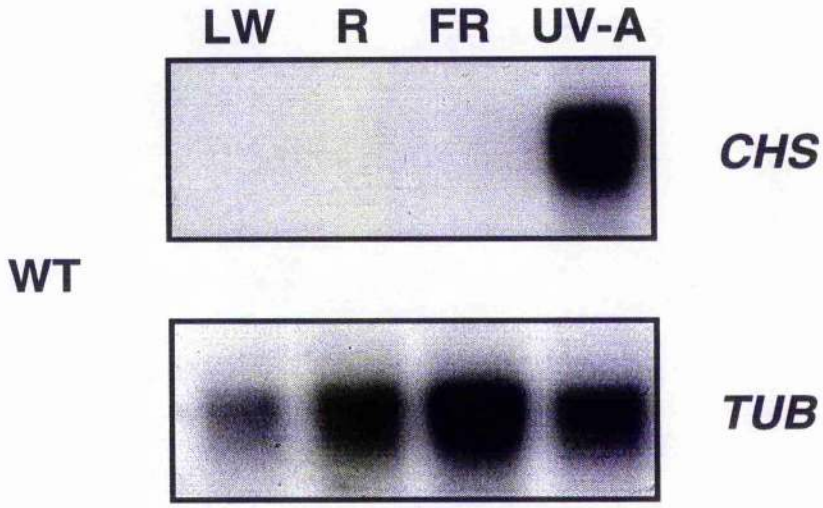


Figure 3.2.6 Wild type *CHS* expression is not increased by red and far red light in mature leaves

Three week old plants were exposed to 6 hours of the indicated light treatments. LW: low white light at 20 µmol $m^{-2} s^{-1}$, R: red light at 65 µmol $m^{-2} s^{-1}$, FR: far red light at 55 µmol $m^{-2} s^{-1}$, UV-A- UV-A light at 80 µmol $m^{-2} s^{-1}$ as a positive control.

Total RNA was extracted from leaves. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

seen. A positive control of a six hour UV-A light treatment at 80 μmol m^{-2} s^{-1} was also included; it can be seen that *CHS* is induced and the lack of signal in the other light qualities is not due to insufficient exposure of the autoradiograph to the radioactively probed filter. These data agree with the previously published information on the induction of *CHS* in mature wild type *Arabidopsis* (Fuglevand *et al.*, 1996; Jackson *et al.*, 1995).

All the above experiments were repeated at least three times and representative northerns are shown. It should be noted that blue light induction of *CHS* was the most variable of the treatments described. The same light fluence levels could produce a range of responses, from a significant induction, to a lesser induction. The reason for the difference in response is unclear as all attempts were made to maintain the same growth and treatment conditions in all repeats of the experiment.

3.2.2 ICX1 Acts Downstream of Several Photoreceptors

icx1 was isolated due to having increased *CHS* transcript levels in response to increasing white light (Jackson *et al.*, 1995). To determine if this was caused by ICX1 acting downstream of a particular photoreceptor, the responses of *icx1* were compared to those of wild type under various light regimes.

The light conditions used by Jackson *et al.* were various fluence rates of white light. If ICX1 were only acting downstream of a particular photoreceptor, for example, cryptochrome 1, an increase in *CHS* expression would only be expected after treatment with Blue/UV-A light and not with UV-B light. If ICX1 does not specifically act downstream from one photoreceptor, an increase in all light qualities which produce an increase in wild type would be expected. It is possible that the increase in *CHS* expression seen in the *icx1* mutants is a result of a loss of regulation by the various photoreceptors. If this were the case, the increased *CHS* expression may be due to red or far red light exposure leading to *CHS* expression as well as the normal blue/UV-A light induction. As described previously (Section 3.2.1), treatment of wild type plants indicated

that the optimum time for maximal induction of *CHS* varied depending on the particular light treatment the plants were exposed to. Time courses of light treatment effects on *CHS* expression were made to ensure that any kinetic differences between the wild type and *icx1* mutant were clear.

3 week old *icx1* and wild type plants were transferred from low white light to different light qualities at a higher fluence rate, and the levels of *CHS* transcript compared. The light qualities that wild type and *icx1* plants were treated with were blue, blue/UV-A, UV-A, UV-B, red and far-red, as in the previous section.

As seen in Figure 3.2.7 treatment with blue filtered light does not produce an obvious difference between the *CHS* expression levels in wild type and *icx1*. Perusal of Figure 3.2.7 indicates that the response to 80 μmol m^{-2} s^{-1} of blue light does not involve ICX1 as no effect is seen in the *icx1* mutant. However, treatment of *icx1* and wild type plants with blue light at fluence rates greater than 100 μmol m^{-2} s^{-1} produce an increase in *CHS* expression in *icx1* greater than that of wild type plants. It should be mentioned again that the blue light induction of *CHS* expression is the most variable in this study.

icx1 plants treated with 80 μmol m^{-2} s^{-1} of blue/UV-A light (Figure 3.2.8) or 80 μmol m^{-2} s^{-1} of UV-A light (Figure 3.2.9) show a greater increase in *CHS* transcript accumulation than that of the wild type. In Figure 3.2.9 a representative northern shows *CHS* expression of wild type and *icx1* plants after 0, 2, 4, 6, 8, 10, 12 and 24 hours of exposure to UV-A light. It can be observed that with the exception of the control 0 hour and the 2 and 24 hour treatments, *CHS* expression in *icx1* is greater than that of the wild type.

Figure 3.2.10 shows a northern blot of three week old wild type and *icx1* plants grown under low white light (20 μmol m^{-2} s^{-1}) and then transferred into 3.3 μmol m^{-2} s^{-1} of UV-B light. Plants were harvested after 2, 4, 6, and 24 hours of UV-B exposure. 0 hour shows the untreated control. As can be seen in Figure

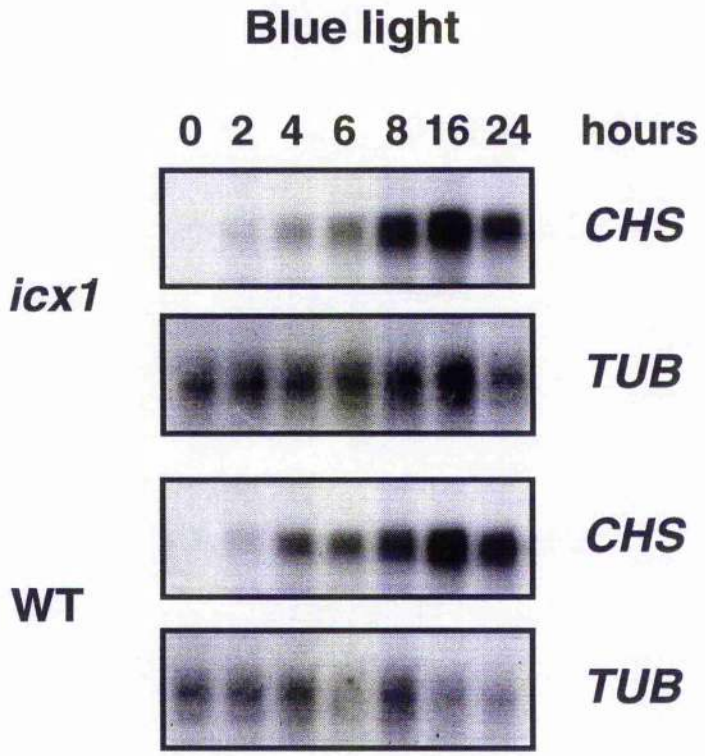


Figure 3.2.7 *icx1* does not show alteration of *CHS* expression in blue light

Three week old plants grown in low white light were transferred into 77 µmol m^{-2} s^{-1} blue light. Plants were harvested after the exposure times indicated and subjected to northern analysis.

Total RNA was extracted from leaf tissue. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of adioactivity and reprobed with an α-tubulin (*TUB*) probe.

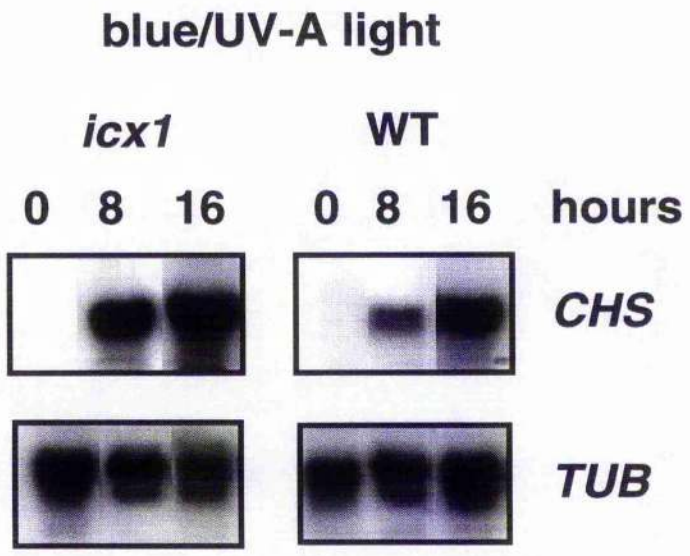


Figure 3.2.8 *CHS* expression in *icx1* and wild type plants after blue/UV-A light exposure

Three week old plants grown in low white light were transferred into 84 µmol $m^{-2} s^{-1}$ blue/UV-A light. Leaves were harvested after the number of hours noted and total RNA was extracted. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

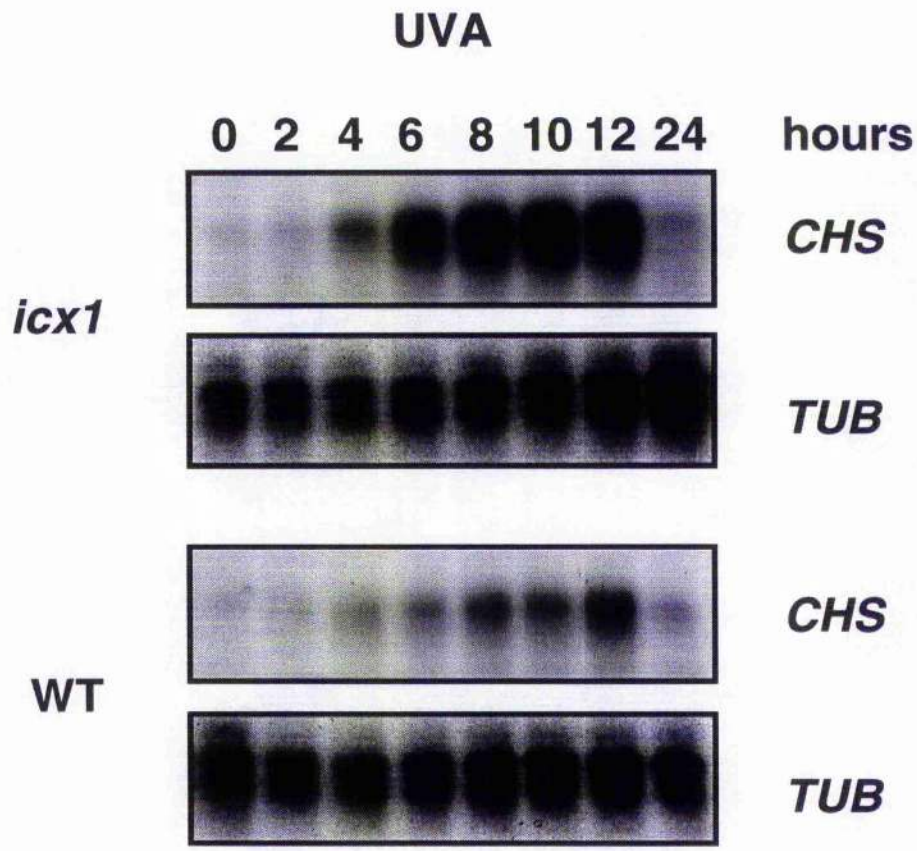


Figure 3.2.9 *icx1* has increased *CHS* expression compared to wild type in UV-A light treatments

Three week old plants were treated with 80 μmol m^{-2} s^{-1} UV-A for the times indicated. Total RNA was extracted from the leaf tissue. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

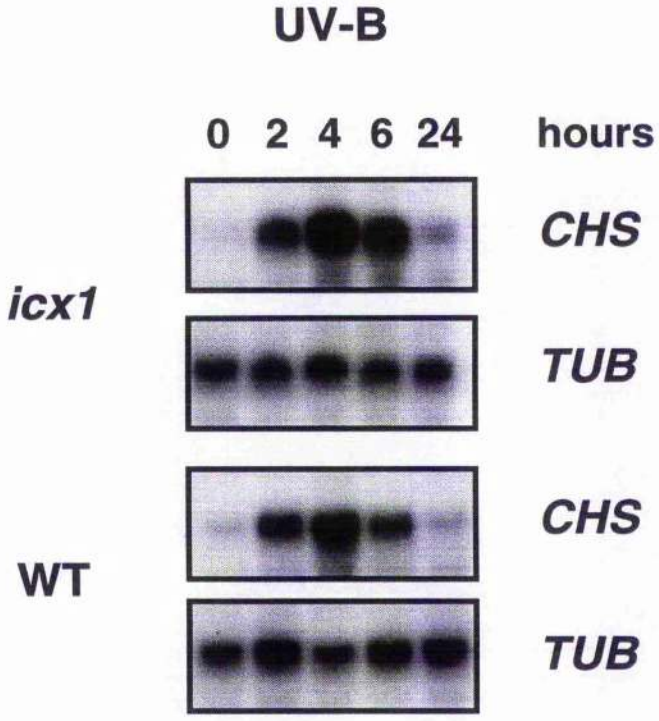


Figure 3.2.10 *icx1* has increased *CHS* expression compared to wild type in UV-B light

Three week old *icx1* and wild type plants were transferred from low white light into 3.3 µmol m^{-2} s^{-1} UV-B light and harvested after the number of hours indicated. Total RNA was extracted. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

This experiment has been carried out more than 10 times with similar results.

3.2.10, *icx1* plants have a level of *CHS* transcript accumulation greater than that seen in the wild type plants after exposure to UV-B light.

The kinetics of *CHS* transcript accumulation in both the wild type and the mutant plants is different in UV-A compared to UV-B light. *CHS* transcript accumulation in wild type and *icx1* plants treated with UV-B increases rapidly, peaking at about 4 hours whereas in UV-A and blue/UV-A light treated plants the *CHS* transcript accumulation reaches a maximum point at about 6-8 hours and remains high for longer. However, the kinetics of transcript accumulation in either UV-A or UV-B light are comparable between *icx1* and wild type; it is the level of accumulation which differs.

In three week old light grown wild type plants, *CHS* is no longer under phytochrome control. Figure 3.2.6 showed that *CHS* transcript expression was not increased in wild type plants exposed to six hours of red or far red light. In the *icx1* mutant plants a similar expression pattern can be seen. *CHS* expression levels of *icx1* and wild type plants in response to red and far red light are shown in Figures 3.2.11 and 3.2.12 respectively. Note that in Figure 3.2.11 the autoradiograph film was over exposed to obtain a visible signal for the *CHS* transcripts. As can be seen in Figure 3.2.11, the *icx1* plants do not show an increase in *CHS* transcript levels above that of the low white control after exposure to red light of 100 μmol m^{-2} s^{-1}. It can be seen that even extended exposure to red light did not produce an increase in *CHS* transcripts in response to red light illumination. The result presented is representative of the numerous repeats made of red light treatment of the *icx1* mutant.

The results from *icx1* plants treated with far red light and subjected to northern analysis are presented in Figure 3.2.12. It can be seen that exposure to 80 μmol m^{-2} s^{-1} of far red light does not produce an increase in *CHS* transcripts above that of the wild type and low white light grown control plants. Control plants were harvested at 0 hours and after 8 hours of high white light (100 μmol m^{-2} s^{-1}). Treated plants were harvested at 2, 4, 6, and 8 hours after exposure to

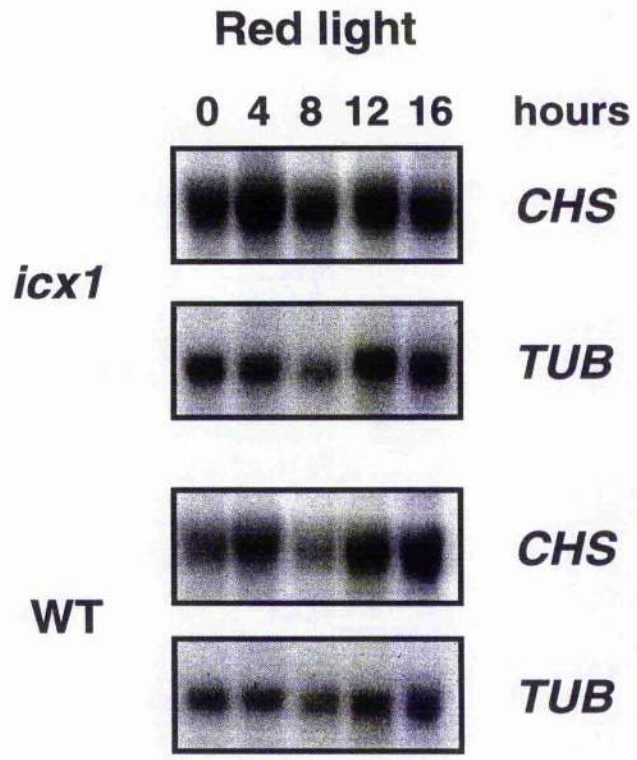


Figure 3.2.11 *icx1* does not show increased *CHS* expression after red light illumination

Three week old low white light grown wild type and *icx1* plants were exposed to 100 µmol m^{-2} s^{-1} red light. Plants were harvested after 0, 4, 8, 12 and 16 hours exposure as indicated, and total RNA extracted. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe.

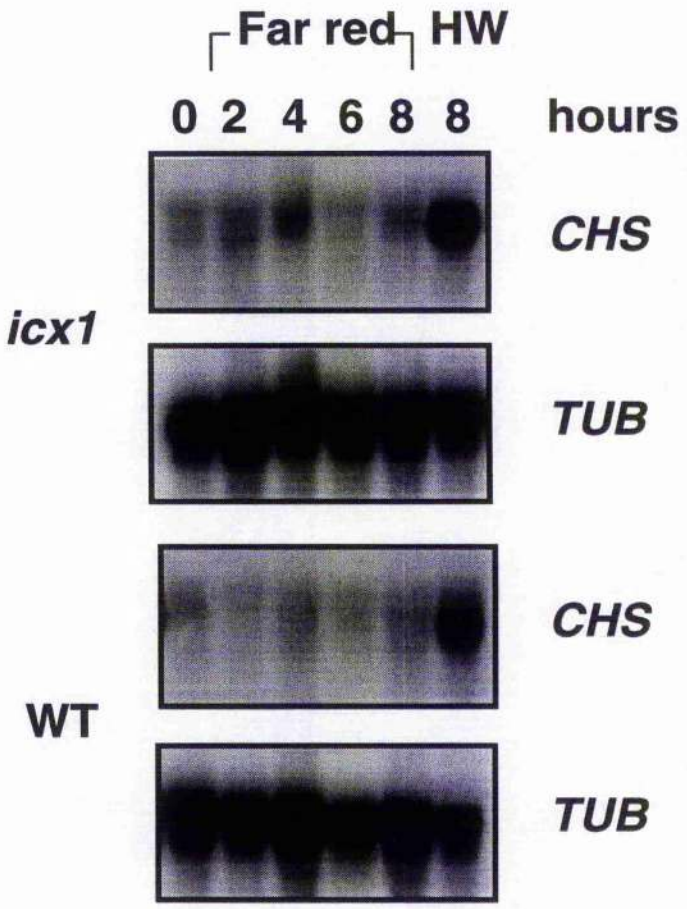


Figure 3.2.12 *icx1* does not have altered *CHS* expression in far red light compared to wild type

Three week old plants were transferred into far red light at 80 µmol $m^{-2} s^{-1}$ for the noted times or into 8 hours of high white light (HW) at 100 µmol $m^{-2} s^{-1}$. 0 is the untreated control.

Total RNA was extracted from the leaves. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

far red light. That far red light does not increase *CHS* transcripts above that of wild type in *icx1* plants indicates that the UV/blue regulation of *CHS* expression is maintained in the *icx1* mutants. Therefore, ICX1 is not involved in phytochrome regulation of *CHS* in three week old, light grown plants.

3.3 ICX1 Acts Downstream of Phytochrome In Dark Grown Seedlings

CHS expression in three week old *Arabidopsis* plants is not regulated by phytochrome (Jackson *et al.*, 1995, see also Section 1.6) and, as seen in Figures 3.2.11 and 3.2.12, this is not altered in the three week old *icx1* mutant plants. However, it is possible to investigate whether ICX1 is involved in signal transduction downstream of phytochrome by studying a different developmental stage of plant growth. In dark grown *Arabidopsis* seedlings, *CHS* expression is regulated by phytochrome until the seedlings are six days old. At this developmental stage *CHS* mRNA can be induced by far red, blue and UV-A light (Kaiser *et al.*, 1995). The effect of UV-B light on flavonoid biosynthetic genes was investigated by Kubasek *et al.* (1992) and found to produce an increase in *CHS* expression in 3 day old dark grown seedlings.

Investigation of the 4 day old dark grown seedlings allows investigation of developmental regulation of *CHS* as well as that by phytochrome control. By comparing the results obtained by light induction of *CHS* in the 4 day old dark grown wild type seedlings with that of the *icx1* mutant any involvement of ICX1 in developmental regulation can be seen.

It has been noted that there are conflicting results in the literature about the induction of *CHS* by various light treatments (Kubasek *et al.*, 1992; Kaiser *et al.*, 1995; Kubasek *et al.*, 1998, see also Section 1.6). Thus, 4 day old wild type seedlings were first tested to investigate *CHS* expression in the experimental set up used here.

Four day old dark grown seedlings were exposed to red, far red, blue/UV-A or UV-B light qualities and total RNA extracted for northern analysis of *CHS* transcript levels in wild type. It can be seen in Figure 3.3.1 that

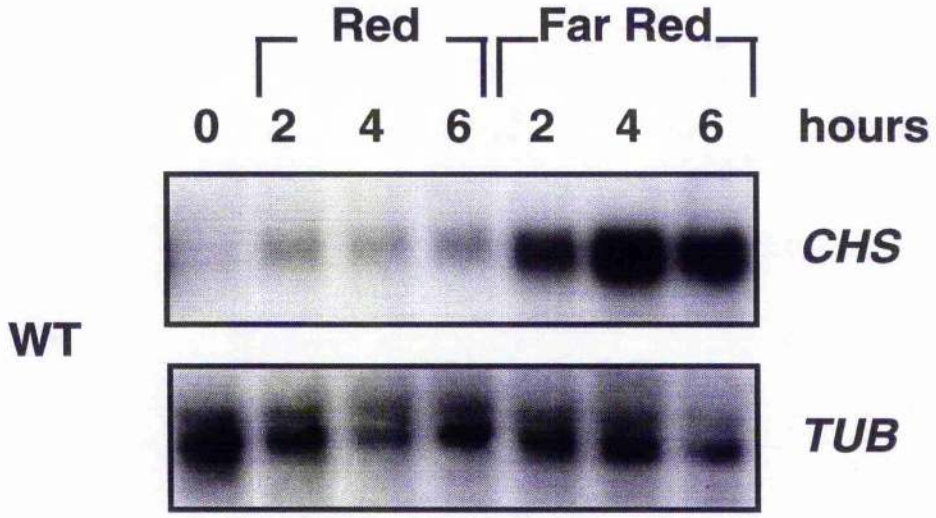


Figure 3.3.1 *CHS* expression in four day old wild type dark grown seedlings illuminated with red and far red light

Four day old dark grown seedlings were treated with 100 μmol m^{-2} s^{-1} red light or 75 μmol m^{-2} s^{-1} far red light for the time indicated. The 0 hour control was harvested within 10 mins of the initial light exposure. Total RNA was isolated from whole seedlings.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

in wild type seedlings, 100 μmol m^{-2} s^{-1} red light does not induce *CHS* transcripts above that of the control. In the same Figure, it can be seen that 4 day old wild type dark grown seedlings do however show an increase in *CHS* transcripts when exposed to 75 μmol m^{-2} s^{-1} of far red light.

It can be noted in Figure 3.3.2 that dark grown seedlings exposed to blue/UV-A light (100 μmol m^{-2} s^{-1}) or to UV-B light (3.1 μmol m^{-2} s^{-1}) displayed an increase in the *CHS* expression levels. This is similar to the induction pattern seen in the three week old plants. Wild type seedlings treated under the experimental conditions used here, show no alteration in the ability or otherwise of *CHS* to be induced in red, blue/UV-A and UV-B light from that of the three week old plants. An alteration does arise in the far red light induction of *CHS* expression in four day old dark grown seedlings. The 4 day old seedlings show an increase in *CHS* expression upon exposure to far red light whereas the three week old plants did not show elevated *CHS* transcript levels.

CHS expression levels in the *icx1* dark grown seedlings treated under the same conditions indicate whether ICX1 is involved in either developmental regulation of *CHS* expression and/or acts downstream from phytochrome in the signal transduction network. If ICX1 is involved in developmental regulation of *CHS* expression, perhaps functioning only in the mature plant tissue, it would be expected that no alteration in *CHS* expression would be seen in the four day old dark grown seedlings compared to the three week old plants. Only the light treatments which produce an increase in *CHS* expression in the wild type dark grown seedlings would produce an increase in *icx1* seedlings, equal to that of wild type.

If ICX1 functions throughout the life of *Arabidopsis*, it would be expected that *icx1* dark grown seedlings would show an increase in *CHS* expression above that of the wild type seedlings. This possibility would allow further investigation of whether ICX1 acts downstream of phytochromes. If ICX1 is acting as a negative regulator in the 4 day old dark grown seedlings, as could

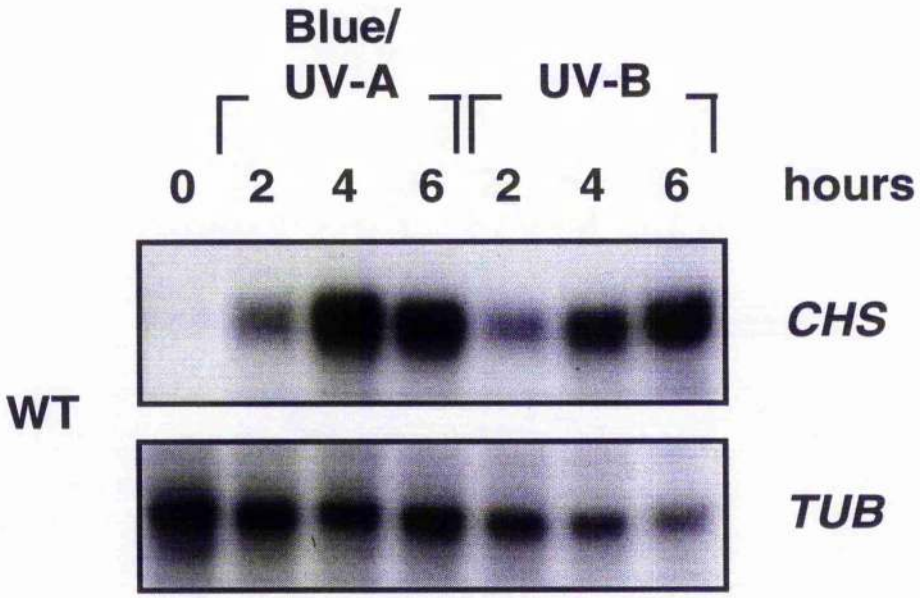


Figure 3.3.2 *CHS* expression in four day old seedlings illuminated with Blue/UV-A and UV-B light

Four day old dark grown seedlings were treated with 100 µmol m^{-2} s^{-1} blue/UV-A light or 3.1 µmol m^{-2} s^{-1} UV-B light for the hours indicated. The 0 hour control samples were exposed to light for less than 10 min during harvesting. RNA was isolated from whole seedlings.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS)* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

be seen by comparison of the wild type and *icx1* blue/UV-A and UV-B treated seedlings, investigation of the relative expression of *CHS* in far red light would indicate if ICX1 acts as a negative regulator downstream from phytochrome. No increase in *CHS* expression in far red light in the *icx1* seedlings from that of the wild type would imply that ICX1 functions as a negative regulator downstream from blue and UV light only. If there is an increase in *CHS* expression in the *icx1* seedlings in far red light, then ICX1 does act as a negative regulator downstream from the phytochromes in four day old dark grown seedlings.

Another possibility is that in dark grown seedlings the lack of *CHS* induction in red light is due to the effect of ICX1. This can be tested in the *icx1* mutant. If ICX1 is itself developmentally regulated, and does not normally function in the dark grown seedlings, the light treatments which induce in the wild type would not be altered in the mutant lacking ICX1.

3.3.1 Developmental regulation

Wild type and *icx1* dark grown seedlings were treated for 2, 4 or 6 hours with either UV-A/blue light (100 μmol m^{-2} s^{-1}) or UV-B light (3.3 μmol m^{-2} s^{-1}) as presented in Figure 3.3.3. The 0 hour control was taken prior to light treatment and harvested within 10 minutes of exposure to light. The four day old *icx1* seedlings showed an increase in *CHS* transcript level above that of the wild type in blue/UV-A, and UV-B light, as the three week old plants had done. This indicates that ICX1 is not developmentally regulated, and still acts as a negative regulator downstream from blue/UV-A and UV-B in the four day old dark grown seedlings.

3.3.2 Phytochrome mediated regulation

Investigation of the red and far red induction of *CHS* expression in the *icx1* mutant will answer the question of whether ICX1 is only involved in UV and blue signal transduction, or if ICX1 also acts in phytochrome mediated signalling. If the expression of *CHS* is not altered from that of wild type in far

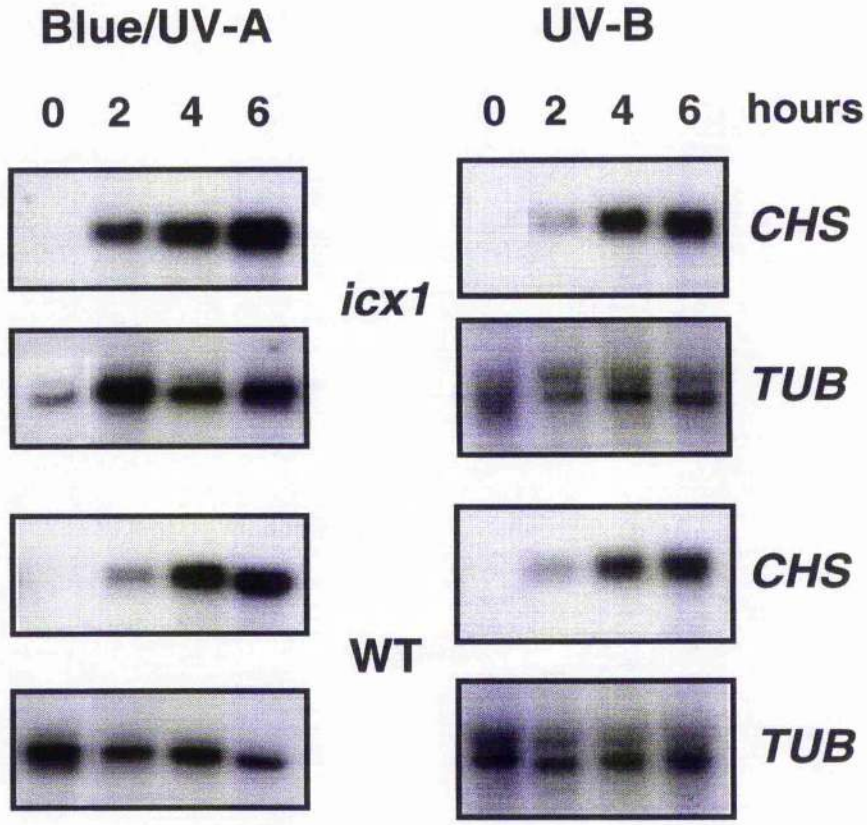


Figure 3.3.3 *CHS* expression in four day old wild type and *icx1* dark grown seedlings illuminated with blue/UV-A and UV-B light

Four day old dark grown seedlings were treated with 100 µmol m^{-2} s^{-1} blue/UV-A light or 3.3 µmol m^{-2} s^{-1} UV-B light for the hours indicated. The 0 hour control samples were exposed to light for less than 10 min during harvesting. Total RNA was isolated from whole seedlings.
Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

red light in the *icx1* mutant, and ICX1 is not developmentally regulated, it could be concluded that ICX1 acts downstream from UV and blue light only.

As ICX1 is acting in the 4 day old dark grown seedling developmental stage (UV-B and blue/UV-A induction, see Section 3.3.1 above), we can observe whether ICX1 is involved in signalling downstream from the phytochromes. 4 day old dark grown seedlings were treated with 100 μmol m^{-2} s^{-1} red light and were harvested after 0 hours (dark control), 2, 4 and 6 hours exposure to the light. As Figure 3.3.4 shows, red light treatment initiated no increase in *CHS* transcript induction over the time course in either wild type or *icx1*, indicating that ICX1 is not involved in the regulation of red light induction of *CHS*.

Also in Figure 3.3.4 dark grown seedlings were treated with far red light, at a fluence rate of 75 μmol m^{-2} s^{-1}. Again the plants were harvested after 2, 4 and 6 hours of light treatment. The 0 hour control was removed from the dark and harvested directly into liquid nitrogen within 10 minutes of initial light exposure. After far red light exposure, it can be seen in Figure 3.3.4, that *icx1* has a greater increase in *CHS* transcript accumulation than wild type. Thus ICX1 must be acting as a negative regulator downstream from phytochrome.

It can be concluded that in four day old dark grown seedlings, ICX1 acts as a negative regulator downstream from phytochrome as well as from the blue/UV-A and UV-B photoreceptors. Phytochrome A has been indicated as the main phytochrome involved in mediating far red light signal transduction in *Arabidopsis* seedlings (Batschauer *et al.*, 1996) so it follows that as *icx1* shows an increase downstream from far red light, ICX1 is acting as a negative regulator downstream from phytochrome A.

3.4 Cryptochrome 1 Expression is Unaffected in *icx1*

Overexpression of cryptochrome 1 in tobacco (Lin *et al.*, 1995a) and co-transfection experiments in *Arabidopsis* (Valentine, 1998) have indicated that the amount of cryptochrome 1 is in fact a rate limiting step in the signal

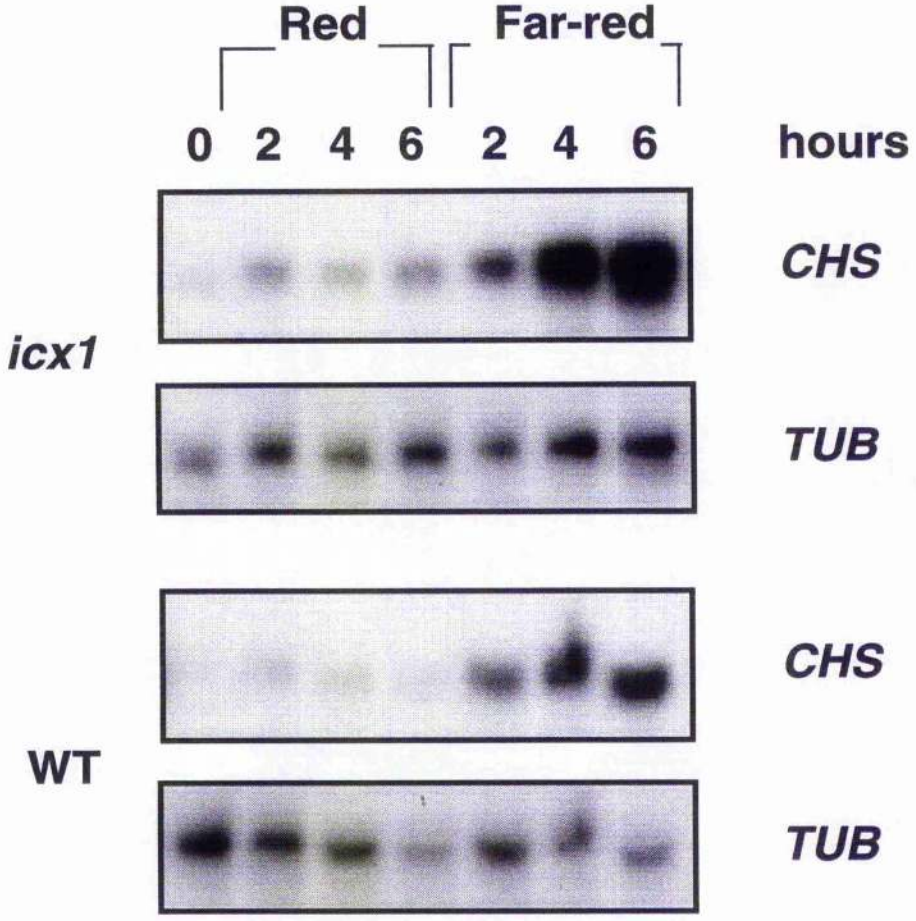


Figure 3.3.4 *CHS* expression in four day old wild type and *icx1* dark grown seedlings exposed to red and far red light

Four day old dark grown seedlings were treated with red light at 100 µmol m^{-2} s^{-1}, or with far red at 75 µmol m^{-2} s^{-1}. Seedlings were harvested after 0, 2, 4, or 6 hours exposure and RNA extracted from the whole seedlings.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

transduction of blue/UV-A light. Increased levels of cryptochrome 1 result in hypersensitivity to blue light. It is possible that the increase in *CHS* transcript induction in *icx1* in blue and UV-A light (but not UV-B) was due to higher levels of cryptochrome-1 (CRY1) in the *icx1* mutant and not due to the mutation of a negative regulatory downstream factor as postulated. If this was the case, levels of *CRY1* messenger RNA from *icx1* would be seen to increase in northerns probed with *CRY1*.

To investigate the possibility of an alteration in the cryptochrome 1 levels in *icx1*, both wild type and *icx1* plants were grown for 3 weeks in non inductive conditions (20 μmol m^{-2} s^{-1}) before light treatment and subsequent northern analysis. In Figure 3.4.1 it can be seen that in *icx1* plants treated for either 6 hours with UV-A/blue light at 80 μmol m^{-2} s^{-1} or for 6 hours with UV-B light at 3.3 μmol m^{-2} s^{-1}, the level of *CRY1* transcript is comparable to that of wild type plants treated in the same conditions. The level of expression of *CHS* transcript is also shown. The apparent absence of a *CHS* transcript signal seen in the UV-B treated plants is due to the length of exposure of the autoradiograph. A longer exposure would produce a greater signal in *icx1* than in the wild type, similar to those results presented above. The result presented is representative of the 3-4 repeats made of this experiment.

This reinforces the model that the increase in *CHS* transcript levels in the *icx1* mutant is due to the removal of a downstream negative regulator rather than because more signal is being channelled into the transduction pathway. This also is consistent with the increase in *CHS* transcript levels seen in UV-B as CRY1 does not absorb in the UV-B wavelengths.

3.5 The Mutation in *ICX1* Increases Flavonoid Biosynthetic Gene Expression but does not Affect the Expression of all Light Regulated Genes

Is ICX1 only involved in the regulation of the flavonoid biosynthetic pathway, or does it affect all light mediated gene expression? Jackson *et al.*

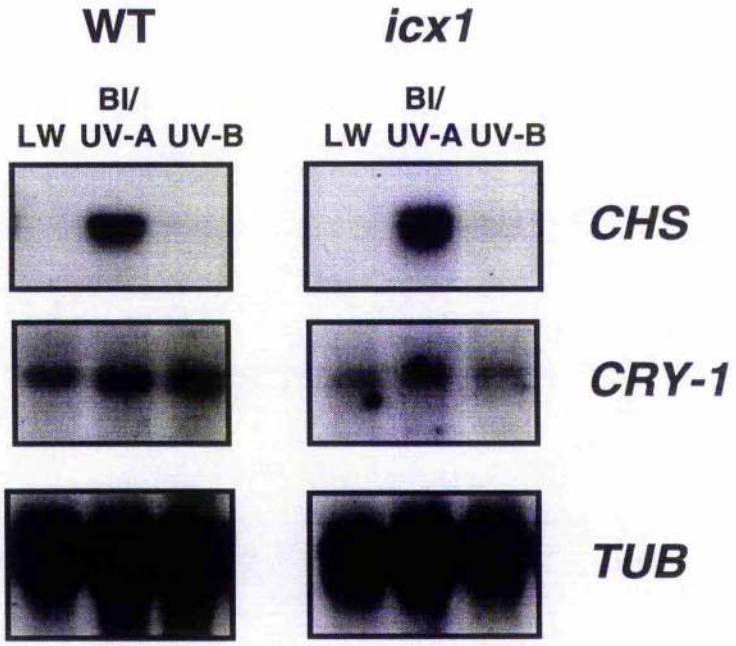


Figure 3.4.1 *CRY 1* expression is not altered from that of wild type in the *icx1* mutant

Three week old plants were transferred into either 6 hours of blue/UV-A light at 80 µmol m^{-2} s^{-1}, (Bl/UV-A) or 4 hours of UV-B light at 3.3 µmol m^{-2} s^{-1} (UV-B). LW is the untreated control.

Total RNA was extracted from the leaves. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an *Arabidopsis* cryptochrome 1 (*CRY1*) probe and an α–tubulin (*TUB*) probe.

(1995) report that in low and high (150 μmol m^{-2} s^{-1}) white light there was an increase in dihydroflavonol reductase (*DFR*) and chalcone isomerase (*CFI*) transcripts in the *icx1* mutant. There was no alteration of *CAB* gene expression between wild type and *icx1* plants in high or low white light.

3.5.1 Effect of the *icx1* Mutation on Gene Expression of Flavonoid Biosynthetic Enzymes

By a combination of analysis of the expression levels of other flavonoid biosynthetic pathway genes and accumulation of downstream products, it can be seen whether removal of ICX1 alters the full flavonoid biosynthetic pathway or whether *CHS* is the only gene with altered expression levels in specific light conditions.

To investigate whether there is a similar increase in *CFI* expression as there is with *CHS* expression in the *icx1* mutant, filters were reprobed with the *CFI* coding sequence. As can be seen in Figure 3.5.1, wild type and *icx1* plants treated with six hours of UV-A light at 80 μmol m^{-2} s^{-1} display an increase in *CFI* transcript expression in the *icx1* mutant above that of the wild type. This indicates that ICX1 acts as a negative regulator in the expression of *CFI* in UV-A light.

The *PAL* sequence was also used to analyse transcript levels in *icx1*. Although the level of PAL transcript detected was low, from Figure 3.5.2 it can be concluded that transcript levels of *PAL* in *icx1* are greater than those of wild type plants treated with 100 μmol m^{-2} s^{-1} blue/UV-A light, 100 μmol m^{-2} s^{-1} UV-A light or 100 μmol m^{-2} s^{-1} white light, as are the levels of *CHS* transcript. Thus, transcription of genes coding for enzymes both upstream (*PAL*) and downstream (*CFI*) from *CHS* in the flavonoid biosynthetic pathway (see also Figure 1.5.1, the flavonoid biosynthetic pathway) are increased in the *icx1* mutant.

Anthocyanin is a product of the flavonoid biosynthetic pathway, it is a

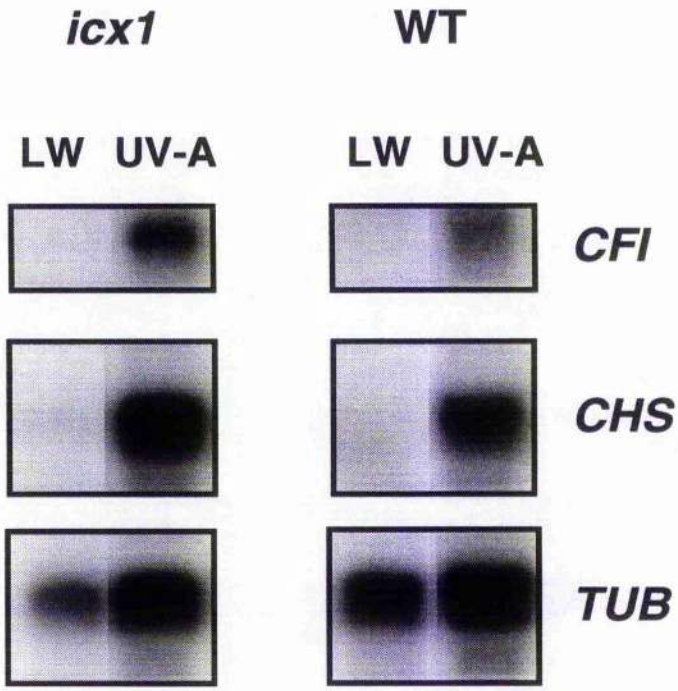


Figure 3.5.1 *CFI* expression in *icx1* is increased compared to that of wild type plants exposed to UV-A light

Three week old plants were transferred into 6 hours of UV-A light at 80 µmol $m^{-2} s^{-1}$ (UV-A). LW is the untreated control.

Total RNA was extracted from the leaves. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with a chalcone isomerase (*CFI*) probe and an α-tubulin (*TUB*) probe.

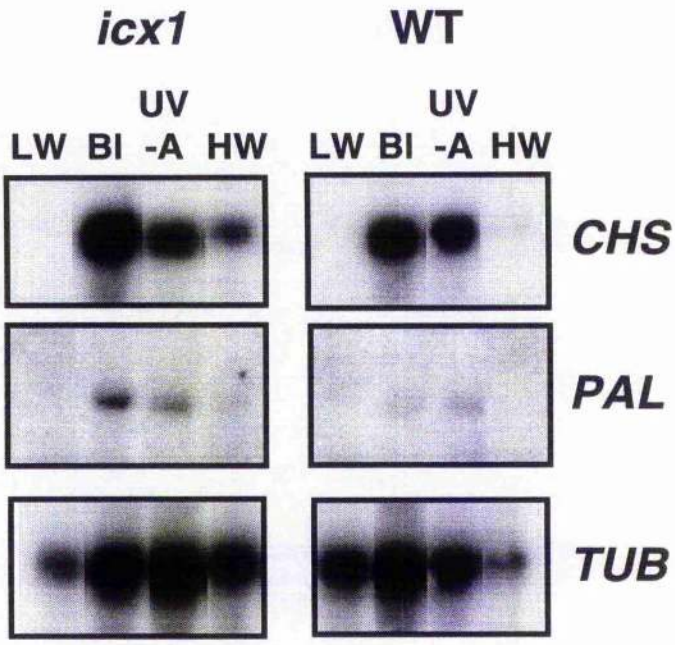


Figure 3.5.2 *PAL* expression is increased in *icx1* plants
Three week old plants were exposed to either 100 µmol m^{-2} s^{-1} blue light (Bl), 100 µmol m^{-2} s^{-1}, UV-A light (UV-A) or 100 µmol m^{-2} s^{-1} high white light (HW). Control plants were not treated and are labelled LW.
Total RNA was extracted from the leaves. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. The membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe. After further stripping of *TUB* probe, the filter was reprobed with a phenylalanine ammonia lyase (*PAL*) probe.

product downstream of PAL, CHS, CFI and DFR enzyme activity. As can be seen in the graph presented in Figure 3.5.3 anthocyanin accumulation in *icx1* is greater in blue, UV-A and UV-B light than in wild type plants. Plants were exposed to 28 hours of either 84 µmol m^{-2} s^{-1} blue, 75 µmol m^{-2} s^{-1} UV-A or 3 µmol m^{-2} s^{-1} UV-B light before assaying for anthocyanin accumulation. The largest accumulation of anthocyanin is in response to blue/UV-A light treatment, followed by that of UV-B light treatment, and UV-A light treatment. For both *icx1* and wild type plants, all three light treatments produce greater accumulation of anthocyanin than in low white light. *icx1* plants are therefore altered in the flavonoid biosynthetic pathway in response to specific light qualities.

3.5.2 *CAB* and *rbcS* Expression in *icx1*

In order to determine whether ICX1 is involved in pathways other than the flavonoid biosynthetic pathway, analysis of the chlorophyll a/b-binding protein (Lhcb;*CAB*) transcript was also completed. CAB is a nuclear encoded protein which acts in the chloroplast in the photosystem II light harvesting system. Unlike *CHS*, which is expressed in the epidermis, *CAB* is expressed in the mesophyll. Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is an enzyme which functions in the chloroplast and catalyses the first step in carbon dioxide fixation in the Calvin cycle and also initiates photorespiration. The small subunit of Rubisco (rbcS) is a nuclear encoded protein which is transported to the chloroplast after synthesis. As with *CAB* expression, *rbcS* expression is mainly confined to the photosynthetically active mesophyll.

As more CAB is required at low light to optimise light capture, *CAB* mRNA transcripts are more abundant in low light and non photosynthetically active light than in high white light. Rubisco shows a light regulated increase in expression levels.

The levels of *CAB* transcript accumulation in the *icx1* and wild type plants were compared under a number of conditions. As presented in Figure

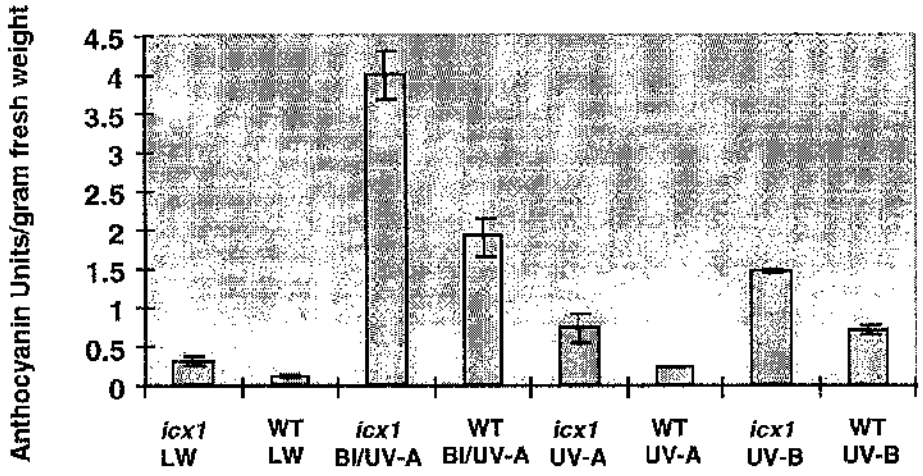


Figure 3.5.3 Anthocyanin accumulation in *icx1* and wild type plants

Three week old *icx1* and wild type plants were transferred from low white light into either 84 µmol m^{-2} s^{-1} of blue/UV-A light, 75 µmol m^{-2} s^{-1} of UV-A light or 3 µmol m^{-2} s^{-1} of UV-B for 28 hours. The control plants remained in low white light (LW).

Anthocyanin was extracted as described in Section 2.17 and is calculated as anthocyanin units per gram fresh weight. Standard error is indicated; where no error bars are shown, standard error is too small to be seen.

3.5.4A, *icx1* and wild type plants both show a decrease in the accumulation of *CAB* transcript levels after 6 hours of 80 μmol m^{-2} s^{-1} blue light or 3.3 μmol m^{-2} s^{-1} UV-B light illumination. There is little difference between the level of *CAB* expression seen in the *icx1* and wild type plants after treatment with any of the light conditions used. In plants exposed to either red or UV-A light no alteration between wild type and *icx1* plants can be seen in *rbcS* expression (Figure 3.5.4B). While both *CAB* and *rbcS* expression levels were altered in the light conditions used, no difference can be seen between the expression levels of *icx1* and wild type plants.

Thus ICX1 is not involved in the regulation of *CAB* or *rbcS* expression, which reinforces the model of epidermal specificity of ICX1. This is consistent with data from Sohal (1997) which indicates that an epidermis-specific lipid transfer protein (LTP) gene is altered in expression in the *icx1* plants.

3.6 ICX1 does not Regulate the Spatial Expression of *CHS*

The increase in accumulation of *CHS* mRNA in response to inductive light treatments could be due to an alteration of spatial expression of *CHS* in the *icx1* mutant. The *det1* mutant shows an increase in the amount of anthocyanins accumulated, which is at least in part due to a loss of tissue specificity of *CHS* expression and anthocyanin accumulation (Chory *et al.*, 1989). Whereas normal expression of *CHS* in the mature leaves of wild type plants is in the epidermis and vascular tissue, the *det1* mutants showed expression of *CHS* (measured using a *CHS-GUS* construct) throughout the leaf tissue, including in mesophyll and stomatal guard cells.

In wild type plants *CHS* is expressed only in the epidermis, if *CHS* was also expressed in the mesophyll tissue in *icx1*, this might account for the overall increase in *CHS* expression. However if *CHS* expression in *icx1* is retained in the epidermis this would indicate that ICX1 is not involved in tissue specificity and reinforces the theory that its role is as that of a negative regulator, removal

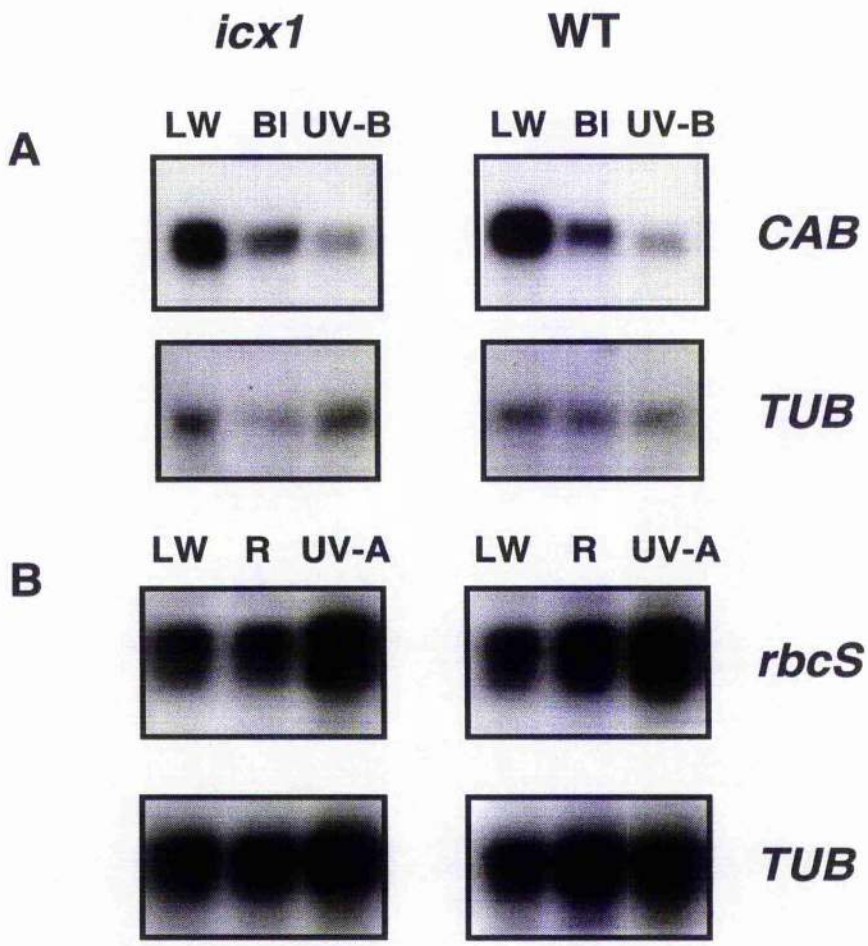


Figure 3.5.4 *CAB* and *rbcS* expression is not altered in *icx1* plants compared to wild type plants

Three week old plants were transferred into (A) 6 hours of blue light at 80 µmol $m^{-2} s^{-1}$, (Bl) or 6 hours of UV-B light at 3.3 µmol $m^{-2} s^{-1}$ (UV-B) or (B) 6 hours of red light (R) at 45 µmole $m^{-2} s^{-1}$ or 6 hours of UV-A light (UV-A) at 80 µmol $m^{-2} s^{-1}$. LW denotes the untreated controls.

Total RNA was extracted from the leaves. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blots were probed with chlorophyll a/b binding protein (*CAB*) probe or ribulose 1,5-biphosphate carboxylase/ oxygenase small subunit (*rbcS*), washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

These experiments have been carried out at least 5 times with similar results.

of which causes an up regulation of *CHS* expression in the epidermis. The evidence that the *icx1* phenotype has multiple alterations in the epidermis (Jackson *et al.*, 1995, J. A. Jackson, R. A. Brown and G. I. Jenkins, unpublished) would support the possibility of an altered level of *CHS* in the epidermis itself.

The spatial expression of *CHS* can be observed via *CHS-GUS* transgene expression in the *icx1* mutant. The *CHS-GUS* transgene used to screen for the *icx1* mutation was lost during back crossing to wild type. That these *icx1* plants back crossed twice to wild type retained the pleiotropic alterations to the phenotype confirms that the presence of the transgene was not the cause of the altered phenotype. Reintroduction of the *CHS-GUS* transgene into the *icx1* plants by crossing allows the spatial regulation of *CHS* to be observed in *icx1* and compared to that of the *CHS-GUS* containing wild type parent line.

icx1 plants were crossed to the *Arabidopsis thaliana* JH10 line, containing the *Arabidopsis CHS* promoter-*GUS* transgene. The F1 seeds were collected and sown out on soil and F2 seeds collected. The F3 generation were screened for the *icx1* phenotype of narrow leaves. Those plants with narrow leaves were subjected to an inductive treatment of 16 hours in high white light. Single leaves from each plant were then tested for the presence of GUS by incubation of the leaf in X-Gluc (as described in Section 2.16). Plants with leaves which produced a blue product were grown on for seed.

The introduction of the *CHS-GUS* transgene into *icx1* enables a direct visualisation of where *CHS* promoter activation is occurring. In the presence of GUS protein, X-Gluc is converted into a water insoluble blue coloured precipitate, dichloro-dibromo-indigo. Incubating plants, or sections of leaves with X-Gluc shows where the *CHS-GUS* is being expressed and the expression pattern can be compared between *icx1* and JH10, the wild type *CHS-GUS* containing line.

CHS-GUS containing JH10 and *icx1* plants were exposed to high white light for 24 hours and leaves were removed, fixed and GUS stained. As Figure 3.6.1 shows, the GUS staining in JH10 and *icx1* is similar, in both cases the

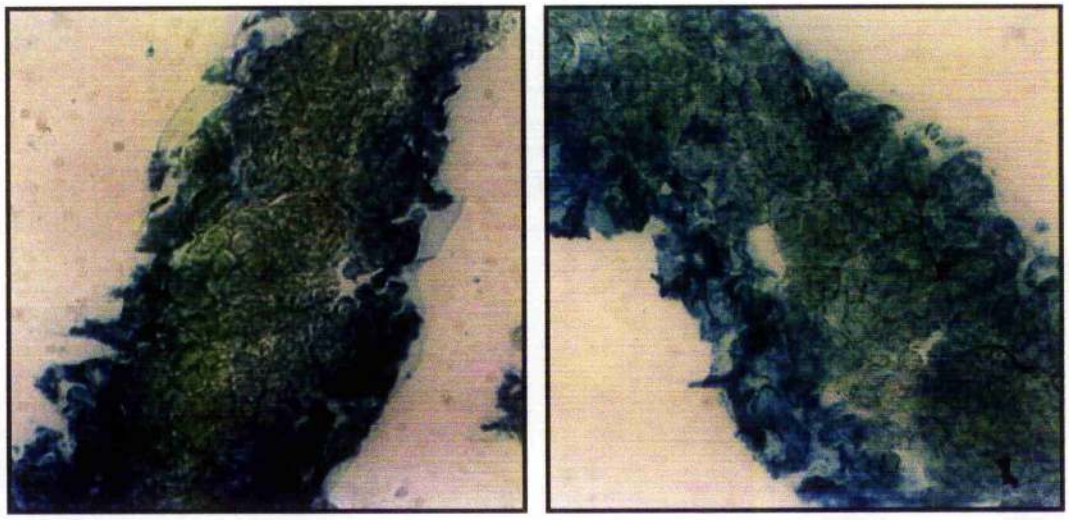


Figure 3.6.1 Spatial expression of *CHS-GUS* in *icx1* and wild type plants
Three week old low white light grown *icx1* and wild type plants were transferred into high white light (100 μmol m^{-2} s^{-1}) for 24 hours prior to fixing and sectioning as described in Section 2.16. Wild type plants are JH10, which contains the *CHS-GUS* transgene. JH10 was crossed with *icx1* to obtain *CHS-GUS* containing *icx1* plants.

blue precipitate is localised in the epidermis. That the spatial extent of the staining in *icx1* is the same as the control, JH10, implies that the increase in *CHS* expression is due to an increase in the amount of *CHS* transcripts produced in the epidermis, and not due to an altered expression pattern of *CHS* in the mutant plants.

3.7 ICX1 does not only Act Downstream of Light Signal Transduction Pathways

Low temperature, low nitrate availability and high sucrose are all known to increase *CHS* transcript levels in *Arabidopsis* (Leyva *et al.*, 1995; Tsukaya *et al.*, 1991). Low temperature is reported to increase both *PAL1* and *CHS* gene expression in *Arabidopsis* (Leyva *et al.*, 1995). This increase is light dependent.

If ICX1 only acts downstream of light mediated signals, *CHS* transcript accumulation would not be altered from that of wild type after cold treatment of *icx1* plants. Similarly, no effect would be seen in plants grown under low nitrate or high sucrose. If *icx1* has altered *CHS* expression in response to a non-light signal it would indicate that ICX1 acts in other signal transduction pathways. If *icx1* remains unaltered from wild type this would indicate that ICX1 only functions in light regulation of *CHS* expression.

3.7.1 Cold Induction of *CHS* Expression

Three week old plants were transferred from low white light at 20°C to the same light quality at 7°C. As can be seen in Figure 3.7.1, wild type plants begin to accumulate *CHS* transcripts initially by about 8 hours after commencing cold treatment, with the amount increasing after 24 hours. After two and three days the level of *CHS* transcript accumulation is still high (data not shown).

When the *icx1* mutant was compared with that of the wild type, *CHS* transcript levels in *icx1* were seen to increase faster than those in the wild type. Figure 3.7.2 is a comparison of *icx1* and wild type after 0, 4, 6 and 8 hours of 7°C

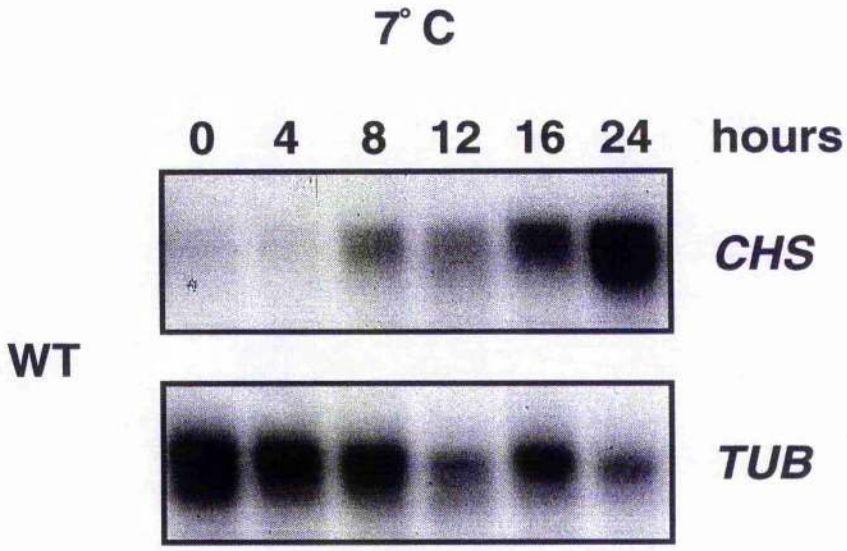


Figure 3.7.1 Cold induction of *CHS* expression in low white light

Three week old wild type plants (WT) were transferred from room temperature, in which they had been grown, to the same light conditions (20 µmol m^{-2} s^{-1}) at 7°C. Plants were harvested at the times indicated after transfer and total RNA extracted.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe.

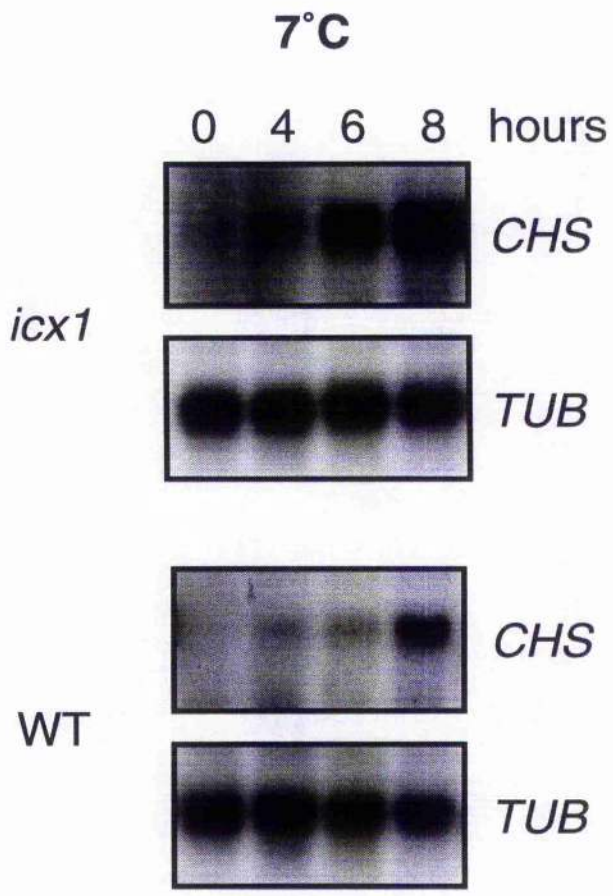


Figure 3.7.2 *CHS* expression in *icx1* and wild type after cold treatment

Three week old low white light grown plants were transferred into the same light quality (20 µmol m^{-2} s^{-1}) at 7°C. Plants were harvested after 0, 4, 6, and 8 hours and total RNA extracted. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

treatment. It can be seen that *icx1* shows an increase in *CHS* transcript accumulation before that of the wild type. By 6 hours of cold treatment a significant accumulation of *CHS* transcripts can be seen in the *icx1* mutant. In the wild type plants the induction is not visible until 8 hours, and that level of induction is not as great as that seen in the *icx1* at the earlier timepoint.

Prolonged treatment in 7°C gives a continued increase in *CHS* expression in both the wild type and *icx1*. After two days of cold treatment *icx1* and wild type both showed greatly increased levels of *CHS* in response to the lowered temperature. By 48 hours of cold treatment, no difference in the levels of *CHS* transcript could be seen between wild type and *icx1* (data not shown).

ICX1 is therefore involved in signal transduction in response to cold and affects the level of *CHS* transcripts. The final level of expression does not, however, seem to be altered in the *icx1* mutant.

The *CHS* transcript accumulation was shown to be light dependent in both the *icx1* and wild type plants, with neither accumulating any observable *CHS* mRNA in a dark plus cold control experiment.

3.7.2 Sucrose Induction of *CHS* Expression

Seeds were plated out on growth media with (+) or without (–) 2% sucrose, vernalised and allowed to germinate in the dark. After four days, no clear difference could be seen between *icx1* seedlings on plates containing sucrose compared to those grown without the presence of sucrose. At four days, the *icx1* plants allowed to germinate in low white light +/– sucrose showed differences; the plants grown on – sucrose were greener, lacking the visible anthocyanin accumulation seen in the plants grown on plates + sucrose.

To determine whether the sucrose levels had an effect on *CHS* transcript accumulation in the *icx1* and wild type plants, total RNA was extracted from four day old seedlings. The seedlings had been germinated in the dark and on day four, exposed to six hours of either low or high white light (Figure 3.7.3). The *icx1* seedlings exposed to either low or high white light in the presence of

sucrose showed an increase in *CHS* transcript accumulation above that seen in the wild type. The level of *CHS* expression seen in low white light was not much higher than that seen in the wild type plants, a larger difference was seen in high white light in the presence of sucrose. As soil grown *icx1* mutants display a higher level of *CHS* expression in response to high white light than wild type, it is possible that there is no additional affect of increased sugar in the *CHS* response of *icx1* mutants.

There was a lower level of *CHS* transcript accumulation in the absence of sucrose than in plants grown on 2% sucrose in both the wild type and *icx1* plants. This is consistent with work done in *Arabidopsis* cell culture (Valentine, 1998) where there was a requirement for sucrose in order for the cells to express *CHS* transcripts in response to inductive conditions. That there is no real difference between the levels of *CHS* expression in *icx1* and wild type plants –S may be due to the ability of the plants to produce sucrose.

It was investigated whether the increase in *CHS* expression seen in *icx1* in the presence of sucrose resulted in a concomitant increase in anthocyanin accumulation. Four day old seedlings which had been grown on plates +/– sucrose were assayed for anthocyanin accumulation, the result of which can be seen in Figure 3.7.4. The *icx1* plants grown on 2% sucrose have at least twice the accumulation of anthocyanin as the wild type plants grown in the same conditions. Both wild type and *icx1* plants grown in the absence of sucrose have much lower levels of anthocyanin accumulation.

icx1 is not altered in its sugar requirement for *CHS* transcript expression, however; in the presence of sucrose, the *icx1* plants show a higher level of *CHS* induction in both low and high white light (Figure 3.7.3). It was also noted that when wild type and *icx1* plants were grown on 2% sucrose in low white light at 20°C the wild type plants grew normally while the *icx1* plants suffered reduced growth, with visible accumulation of anthocyanin and pale

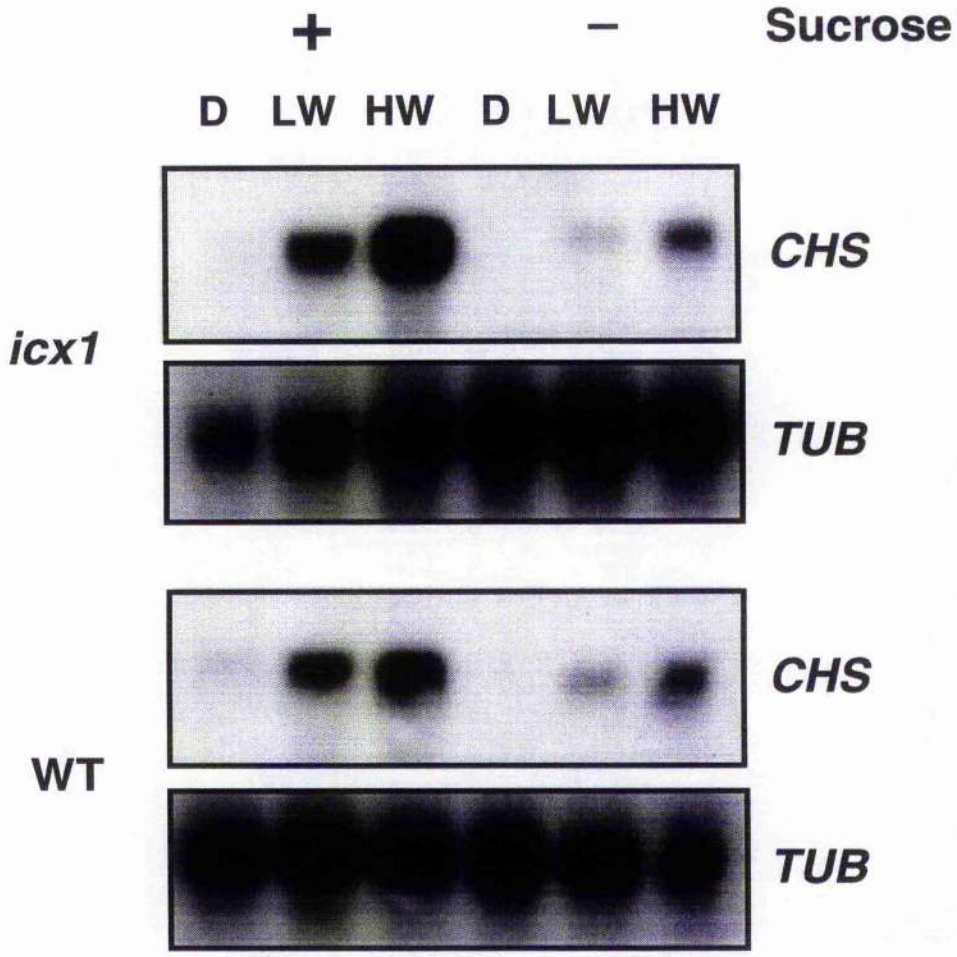
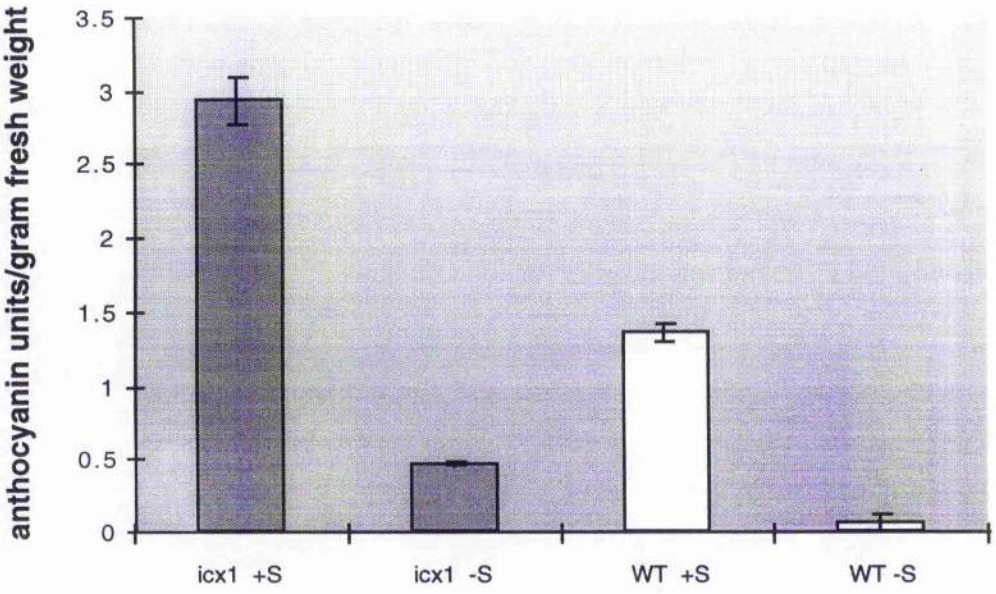


Figure 3.7.3 *CHS* expression in *icx1* and wild type plants grown on 0 or 2% sucrose

Four day old dark grown seedlings grown on either 0 or 2% sucrose were transferred into 6 hours of low white light (LW, 20 µmol $m^{-2} s^{-1}$) or 6 hours of high white light (HW, 100 µmol $m^{-2} s^{-1}$). Untreated plants remained in the dark for the duration of the light treatments (D). The seedlings were harvested into liquid nitrogen.

Total RNA was isolated from the frozen tissue. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.



3.7.4 Anthocyanin accumulation +/- sucrose in wild type and *icx1* seedlings

Wild type and *icx1* seeds were sown on solid growth media containing 0% (-S) or 2% (+S) sucrose. After one hour exposure to low white light and three day vernalisation plants were transferred into high white light (100 µmol m^{-2} s^{-1}) for four days. Tissue was weighed and anthocyanin extracted. Anthocyanin units were calculated per gram fresh weight. Standard error is indicated.

green leaf colour. *icx1* plants grown on – sucrose or on soil did not display any of these growth habits.

It was investigated whether the altered growth of *icx1* on sucrose was a result of an inability to regulate sugar accumulation. This was measured via the total sugar (glucose, sucrose and fructose) present in 4 day old seedlings grown + or – sucrose. Wild type and *icx1* seeds were sown on petri dishes containing medium either with or without 2% sucrose as before. Following vernalisation, the seeds were either transferred into environment controlled growth rooms at 20°C in darkness (DD), or low white light (LW). A further set of plates was transferred initially into darkness then exposed to low white light for 6 hours (D LW) immediately before harvesting. Seedlings were harvested, weighed and surface washed to remove any sugars remaining on their epidermis from the growth media prior to assaying (described in Section 2.18). The amount of sugar was calculated per gram fresh weight for each set of seedlings. The results are presented in Figure 3.7.5. The accumulation of sugars in wild type and *icx1* plants grown on media without sucrose can be seen to be very low for both wild type and *icx1* seedlings, irrespective of the light conditions. However, in *icx1* seedlings grown on 2% sucrose there is a much larger accumulation of sugars than in the wild type seedlings. This is particularly noticeable in seedlings transferred from dark into low white light. It is possible that *icx1* plants grown on sucrose show impaired growth as a result of an inability to regulate sucrose influx, which could then affect a number of aspects of growth. Increased sugars could affect the osmotic balance of cells resulting in the poor growth of *icx1* plants on sucrose.

3.8 Where does ICX1 Act with Respect to Other Known Proteins involved in Light Signal Transduction?

ICX1 regulates *CHS* transcript levels after induction by various light fluences. It appears to act as a negative regulator, downstream of several photoreceptors. But where does it act? We can begin to answer this question by

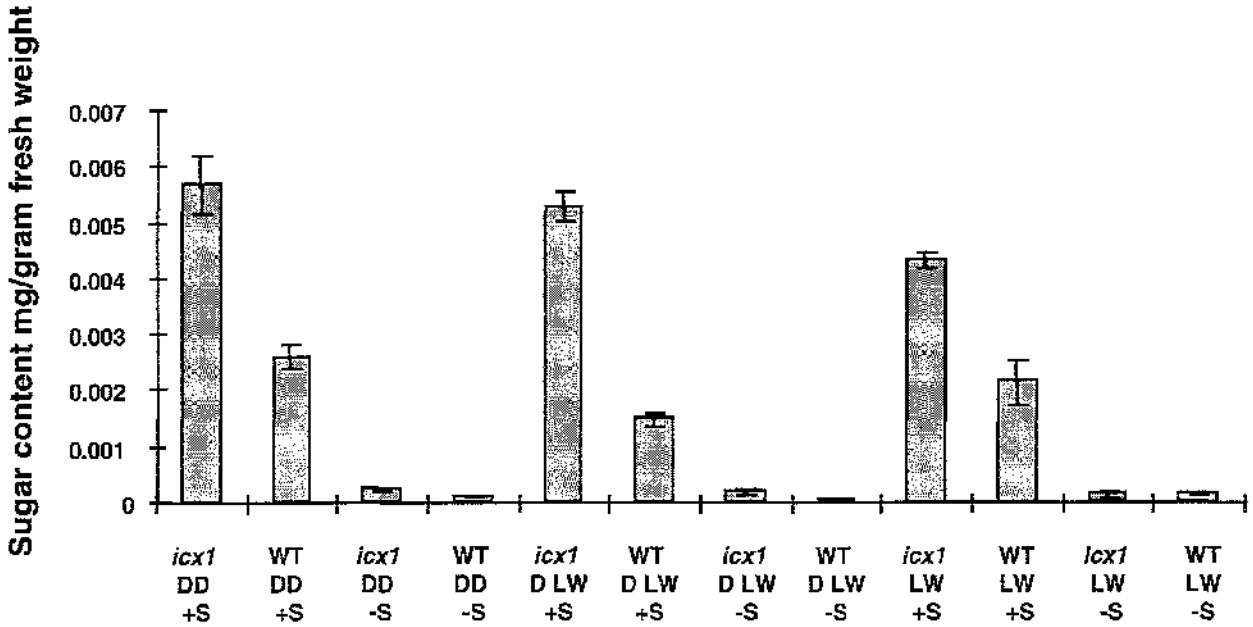


Figure 3.7.5 Total sugar content in 4 day old *icx1* and wild type seedlings.
Seedlings were grown on growth media containing 0% sucrose (-S) or 2% sucrose (+S) in either total darkness, DD, or transferred from dark into light for 6 hours prior to harvesting, D LW, or grown in low white light, LW.
Sugar (glucose, fructose and sucrose) content of the seedlings is calculated per gram fresh weight. Standard error is indicated, where no error bars are shown error was too small to be drawn.

placing ICX1 relative to other known light signal transduction proteins.

Using double mutants for epistasis studies, it is possible to ascertain where ICX1 acts with respect to other proteins. By piecing a number of these results together, a map of where ICX1 acts relative to these proteins in the signal transduction cascade can be produced.

3.8.1 Cryptochrome 1 and ICX1 Epistasis

We know that ICX1 acts downstream of blue/UV-A and UV-B light. The cryptochrome 1 (CRY1) photoreceptor absorbs light in the blue/UV-A wavelengths. The *hy4* mutant lacks cryptochrome, and as a result shows no induction of *CHS* by UV-A light and a lower level of *CHS* induction by blue light (Fuglevand *et al.*, 1996). A second, similar, protein CRY2, may be responsible for the residual blue light responses seen in the *hy4* mutant plants. Currently, no one has isolated a UV-B photoreceptor, though much indirect evidence indicates the existence of at least one UV-B photoreceptor (see Section 1.2.4).

Figure 3.8.1 shows the reduction in *CHS* expression seen in *hy4* in 120 μmol m^{-2} s^{-1} blue light and 80 μmol m^{-2} s^{-1} UV-A light compared to wild type. It has already been noted that the increase in *CHS* expression in *icx1* is not due to an increased level of CRY1. To determine whether ICX1 acts downstream of cryptochrome 1, double mutant studies were carried out using the *hy4-2.23N* null mutant. The *hy4-2.23N icx1* double mutant, *icx1* and *hy4-2.23N* single mutants and wild type were treated with UV-A light and high white light and compared. The *hy4-2.23N* mutant lacks cryptochrome 1 and therefore does not show increased levels of *CHS* transcript in response to UV-A illumination of 80 μmol m^{-2} s^{-1}. Both the wild type and to a greater extent, *icx1*, show an increase in the levels of *CHS* transcript after the same UV-A illumination. As seen in Figure 3.8.2, the *CHS* transcript level in the double mutant was very low, comparable to the level seen in the *hy4-2.23N* mutant. This implies that ICX1 is is acting in the same pathway as HY4 and therefore, as HY4 is acting at the

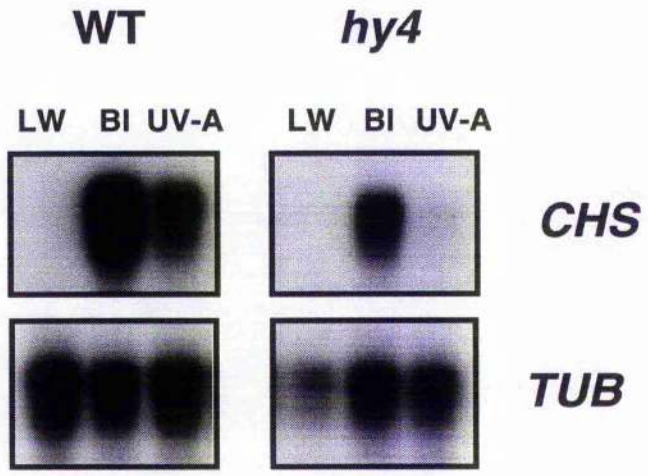


Figure 3.8.1 *hy4* plants have reduced *CHS* expression in response to blue and UV-A light compared to wild type plants

Wild type (WT) and *hy4-2.23N* mutants were grown in 20 µmol m^{-2} s^{-1} of low white light for three weeks. Plants were exposed to 80 µmol m^{-2} s^{-1} UV-A light (UV-A), or 120 µmol m^{-2} s^{-1} blue (Bl) light for 6 hours or harvested without light treatment (LW).

Total RNA was extracted from plant leaves. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

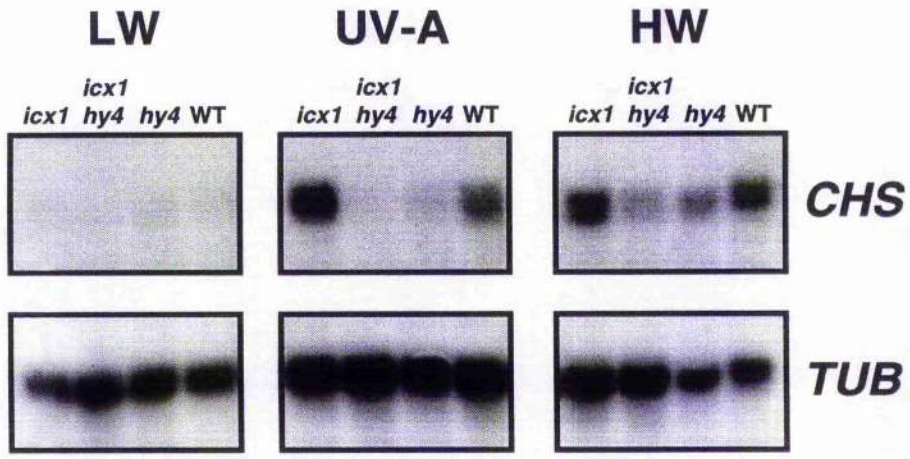


Figure 3.8.2 *CHS* expression in the *hy4-2.23N icx1* double mutant exposed to UV-A and high white light

Three week old low white light grown *hy4-2.23N icx1* double mutant, *icx1* and *hy4* single mutant and wild type plants were transferred into 100 µmol $m^{-2} s^{-1}$ of either UV-A or white light for 6 hours. Plants were harvested and total RNA was extracted from leaf tissue. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

point of absorption of the light signal, ICX1 is acting in the signal transduction pathway leading from HY4 to *CHS* expression. This agrees with the observation that ICX1 has been shown to act as a negative regulator in the UV-A signal transduction pathway (Figure 3.2.8) so it would be expected to act further downstream than the photoreceptor. It is likely that ICX1 also affects the signal transduction pathway downstream of other blue/UV-A light receptors, such as CRY2, although this remains to be tested.

3.8.2 HY5 and ICX1 Epistasis

Another light signal transduction protein is HY5, which is a basic leucine zipper (bZIP) DNA binding protein (Oyama *et al.*, 1997). HY5 is thought to interact with COP1 in the nucleus (Ang and Deng, 1994). *hy5* does not show inhibition of hypocotyl elongation mediated by red, far-red or blue light, and thus HY5 is thought to act downstream of the red, far-red and blue light photoreceptors in this response. *hy5* has been shown to have decreased anthocyanin accumulation in high white light compared to wild type (Chory, 1992).

The induction of *CHS* transcript accumulation in the *hy5* mutant after exposure to UV-A and UV-B is shown in Figures 3.8.3 and 3.8.4. It can be seen that after exposure to UV-A or UV-B light no increase in *CHS* transcript accumulation above that of low white light can be detected. This was repeated numerous times, and in all cases *CHS* induction could not be detected in the UV-A or UV-B illuminated *hy5* plants.

It is possible that ICX1 and HY5 interact in the blue/UV-A and UV-B light signal transduction pathways. Reports that HY5 directly interacts with *CHS* to increase transcript accumulation indicate that where CRY1 is the start of the chain of signal transduction, HY5 is one of the end points (Ang *et al.*, 1998). ICX1 may function as a negative regulator of HY5, or act as a negative regulator independently of HY5.

As ICX1 and HY5 are both involved in the signal transduction of UV-A

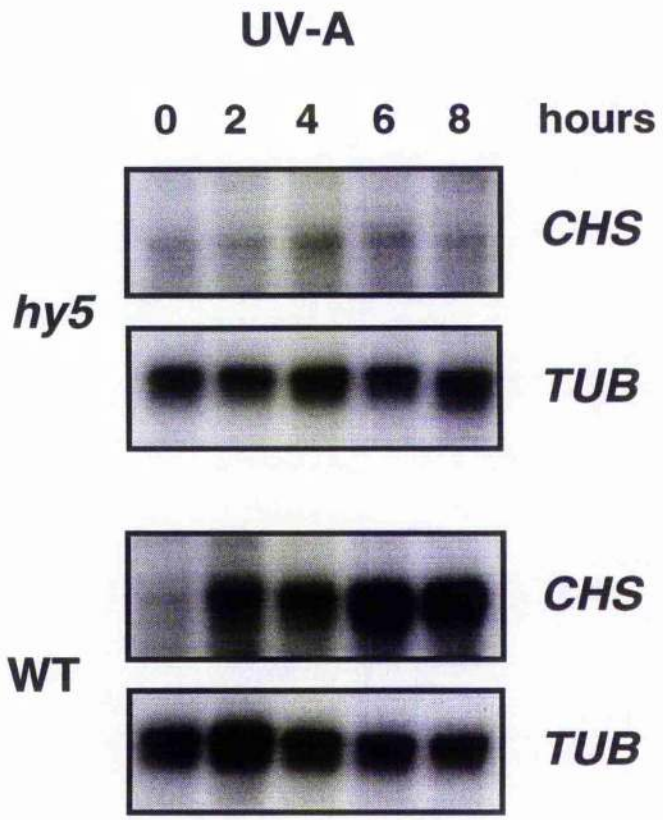


Figure 3.8.3 *CHS* expression after UV-A light treatment in wild type and the *hy5* mutant

Three week old wild type (WT) and *hy5* plants were grown in low white light. After transfer to 90 µmol $m^{-2} s^{-1}$ UV-A light plants were harvested at the times indicated. 0 hour treatment indicates an untreated control.

Leaf tissue was harvested and total RNA extracted. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

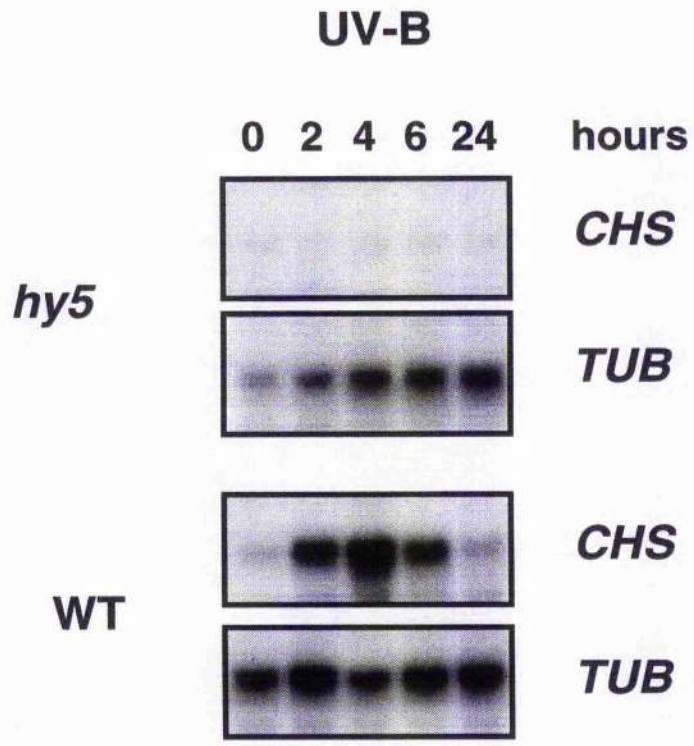


Figure 3.8.4 *CHS* expression in *hy5* plants exposed to UV-B light

Three week old *hy5* and wild type plants were transferred from low white light into 3.3 µmol $m^{-2} s^{-1}$ UV-B light and harvested after the number of hours indicated. Total RNA was extracted from leaves. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

induced gene expression, investigation of the responses of the *hy5 icx1* double mutant, compared to that of the single mutant parents, would be able to indicate whether one gene product is epistatic to the other. If HY5 is acting in the same pathway as ICX1, no accumulation of *CHS* transcripts would be seen. If ICX1 acts independently of HY5, expression of *CHS* transcripts would be expected to be greater than that of wild type.

As can be seen in Figure 3.8.5 the accumulation of *CHS* in the *hy5 icx1* double mutant after exposure to UV-A or UV-B is virtually indistinguishable from that of the *hy5* single mutant parent. To clarify the response to UV-B light, a further filter is shown in Figure 3.8.6. X-ray film was exposed to a radioactively probed northern for longer to allow clearer visualisation of *CHS* expression in response to UV-B light. It can be seen that there is no *CHS* expression in the *hy5 icx1* double mutant in response to UV-B illumination. It would appear that HY5 is required for induction of *CHS* in response to UV-A and UV-B light.

HY5 is essential for expression of the mustard *CHS1* minimal promoter in white light (Ang *et al.*, 1998). It may be that HY5 is required as a positive regulator for *CHS* transcript expression in UV-A and UV-B light, and in the absence of HY5, *CHS* expression is not detectable under UV-A and UV-B light. This does not, however, give any indication of the relative positions of the ICX1 and HY5 gene products in their interaction in the light signal transduction pathways. That HY5 has been shown to interact directly with the *CHS* promoter (Ang *et al.*, 1998) implies that ICX1 could act either upstream of HY5 or at the same point in the signal transduction pathway.

hy5 plants were initially isolated as a result of having reduced hypocotyl inhibition in blue, red and far red light (Koornneef *et al.*, 1980). *icx1* is not affected in hypocotyl length inhibition, though the overall length of *icx1* hypocotyl is shorter than that of wild type. To further investigate the interaction of ICX1 and HY5, hypocotyl lengths of 6 day old *hy5 icx1* double mutant seedlings and the single mutant parents grown in blue light (33 µmol

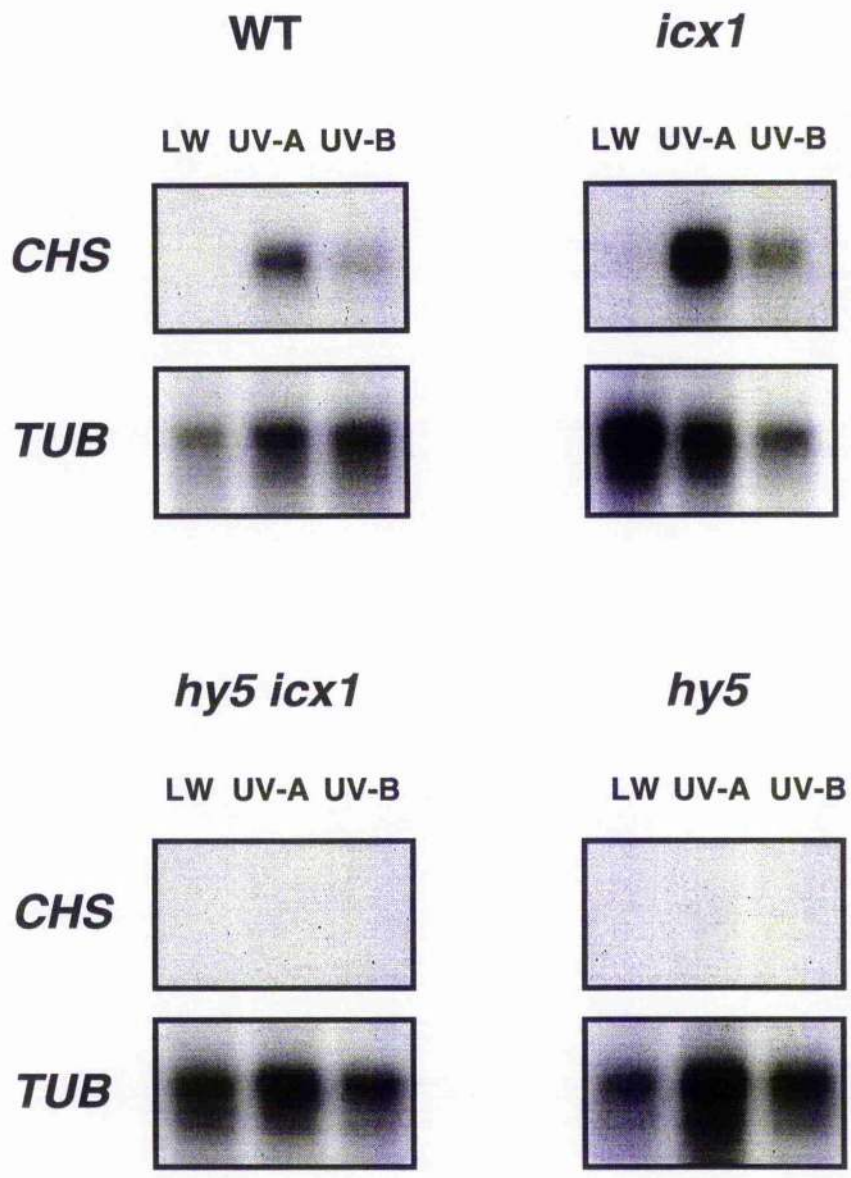


Figure 3.8.5 *CHS* expression in *hy5 icx1* plants exposed to UV-A and UV-B light

Three week old *hy5 icx1* double mutant, *icx1* and *hy5* single mutants and wild type plants were transferred into either 6 hours of UV-A light at 80 µmol m^{-2} s^{-1}, (UV-A) or 4 hours of UV-B light at 3.3 µmol m^{-2} s^{-1} (UV-B). LW is the untreated control.

Total RNA was extracted from the leaves. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

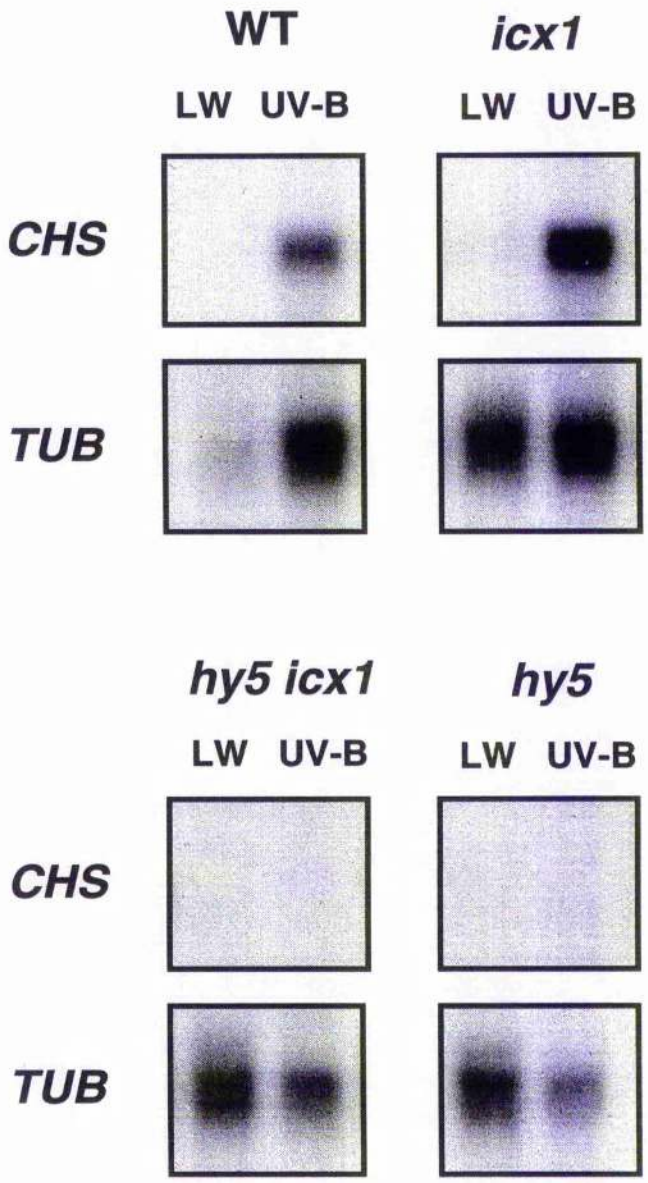


Figure 3.8.6 *CHS* expression in *hy5 icx1* double mutant plants in UV-B light

Three week old *hy5 icx1* double mutant, *hy5* and *icx1* single mutants and wild type plants were transferred into 4 hours of UV-B light at 3.3 µmol m^{-2} s^{-1} (UV-B). LW is the untreated control. Total RNA was isolated from the leaf tissue. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

m^{-2} s^{-1}) were measured and compared to the hypocotyl lengths of dark grown seedlings. This was done to determine whether ICX1 acts downstream from HY5 in blue light to regulate hypocotyl length.

As Figure 3.8.7 shows, *icx1* has a shorter hypocotyl than wild type and *hy5* plants in both conditions, this suggests that *icx1* may be generally affected in extension growth. As expected, in blue light the *hy5* hypocotyls were longer than those of the wild type (Figure 3.8.7A). The hypocotyl length of the *hy5 icx1* double mutant seedlings grown in 33 µmol m^{-2} s^{-1} blue light was intermediate between those of the *icx1* and *hy5* single mutant seedlings. This would imply that *icx1* and *hy5* are not epistatic in the blue light mediated hypocotyl inhibition pathway.

To summarise, ICX1 acts downstream from cryptochrome, but also from another blue light receptor, as a low level of induction of *CHS* transcripts can be seen in the *hy4 icx1* double mutant. ICX1 does appear to be acting in the same pathway as HY5 downstream from UV-A or blue light, as the low level of *CHS* transcripts in the *hy5 icx1* double mutant is similar to that of the *hy5* parent. It would also appear that ICX1 and HY5 are acting in the same pathway downstream from UV-B light. A model for ICX1 interaction in light signal transduction cascades incorporating HY5 and CRY1 is presented in Figure 3.8.8. However with regard to hypocotyl elongation, there is no clear epistasis, and HY5 and ICX1 may be acting independently.

3.9 Involvement of ICX1 in the UV-B/Blue and UV-B/UV-A Synergistic Responses

The positive interaction of inductive signals can be in an additive form or in a synergistic form. Additive interaction of two signals leads to an induction which is equal to the sum of the two separate inductive responses. A synergistic interaction leads to a level of inductive response which is greater than the sum of the parts. By measuring GUS activity in *CHS-GUS* containing plants, Fuglevand *et al.* (1996) investigated the interaction of blue and UV-B or

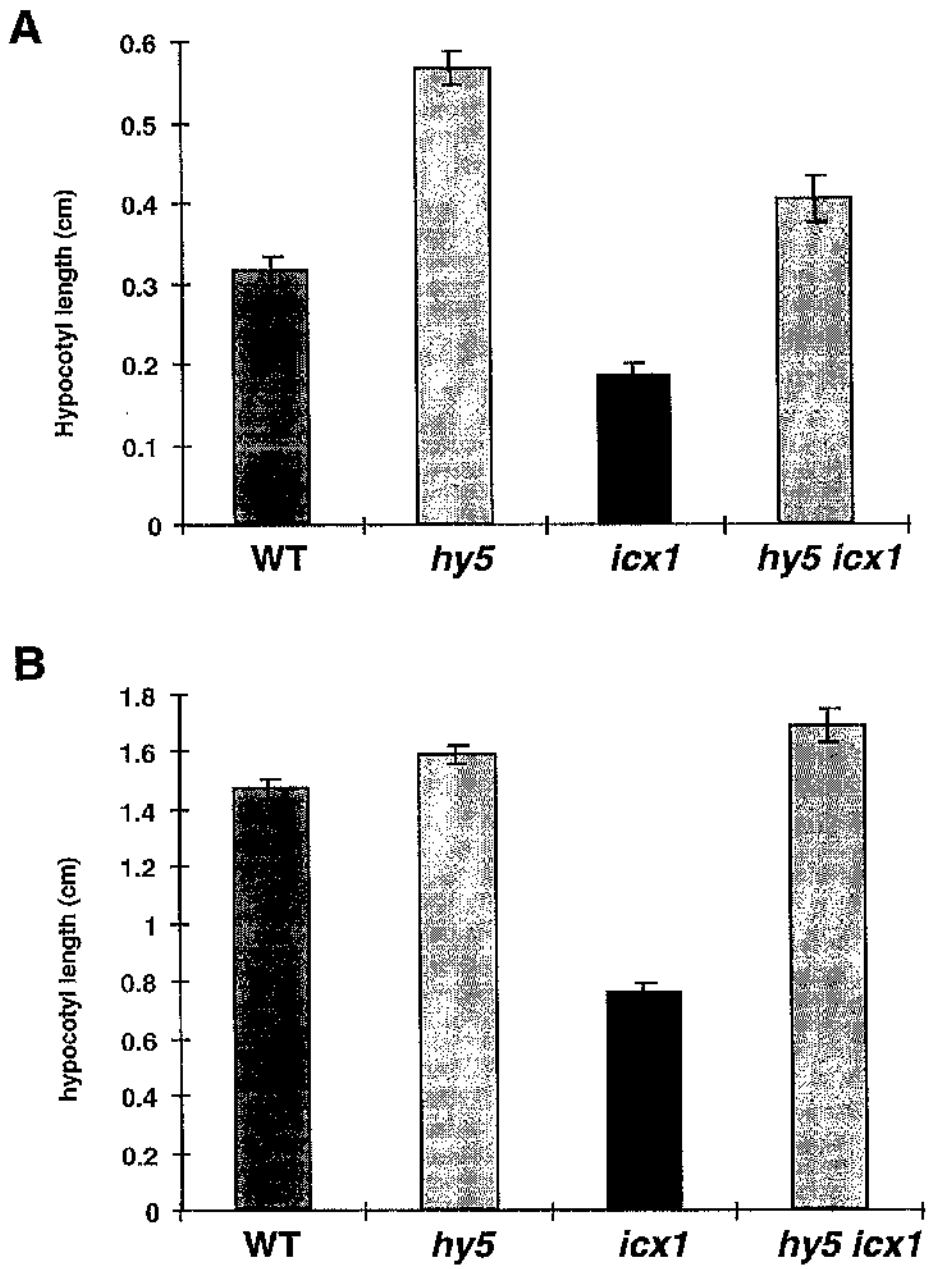


Figure 3.8.7 Hypocotyl length in *hy5 icx1* double and single mutants

hy5 icx1 double mutants, *hy5* and *icx1* single mutants and wild type seeds were sown on soil and vernalised. After vernalisation the pots of seed were transferred to either A) blue light at 33 µmol m^{-2} s^{-1}, B) a dark growth room. After 6 days hypocotyl lengths of 30 plants was measured for each line and the average length was calculated. Standard error is given for each sample.

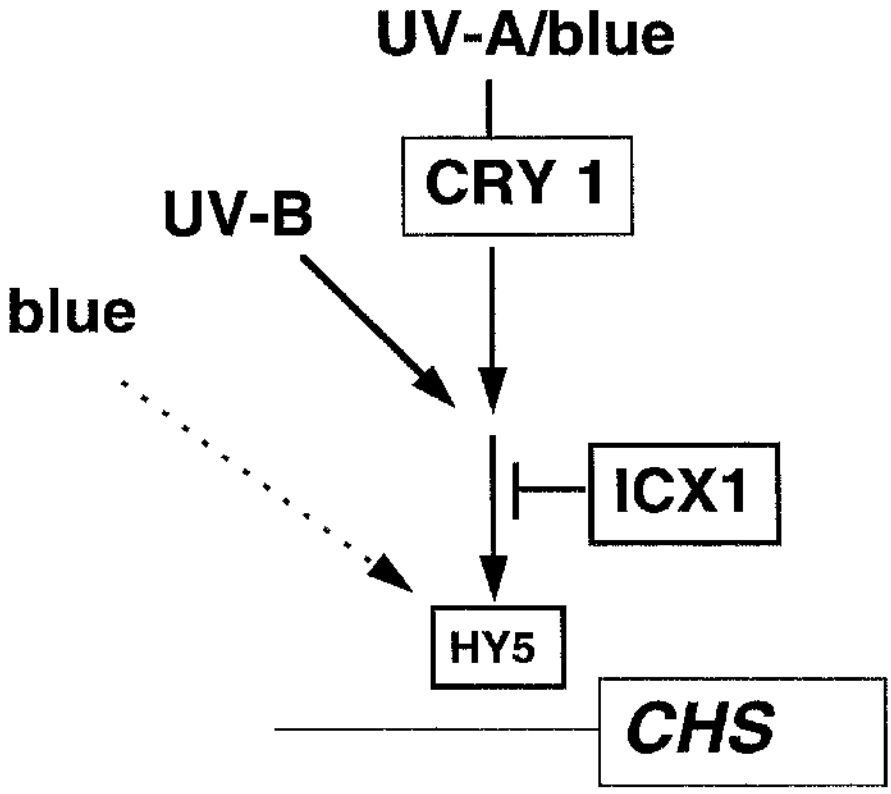


Figure 3.8.8 Model of ICX1 and HY5 interaction in light signal transduction cascades

UV-A and UV-B light on *CHS* expression. The results indicated that the induction of *CHS* after UV-B and blue light illumination is about four times greater than the sum of the two single light responses. A synergistic interaction is taking place in the *CHS* response to UV-B and blue light together. A synergistic level of *CHS* expression was also seen when plants were illuminated with UV-A and UV-B light together. Synergism is explained in greater detail in Section 1.7 and Figure 1.7.1 is of a model of blue/UV-B and UV-A/UV-B synergistic interactions.

From the above study, which investigated the involvement of cryptochrome-1 in the UV-B/UV-A and UV-B/blue synergistic responses, it is thought that there are separate UV-A and blue phototransduction pathways which interact with UV-B in a synergistic manner. The UV-A and blue light signals interacting with UV-B light signals were distinct, as a blue light treatment resulted in a stable signal which, while degraded in the dark, survived 5 hours in the dark prior to UV-B treatment to produce a synergistic *CHS* response. The UV-A light treatment produced a transient signal. UV-A treatment required to be concomitant with that of UV-B or no synergism was seen.

The data presented by Fuglevand *et al.* (1996) were based on a *CHS-GUS* reporter fusion, though expression levels of endogenous *CHS* were found to be comparable with results obtained by GUS assay. Northern analysis of wild type plants to investigate *CHS* expression in response to both UV-B/UV-A and UV-B/blue inductive signals, in comparison to that of the individual light treatments is presented here. Figure 3.9.1 shows a representative northern of *CHS* expression in wild type plants after blue, UV-A, UV-B light exposure, and after UV-B/blue and UV-B/UV-A synergistic light treatment. It can be seen that in order to avoid over exposure of the film in the lanes containing the UV-B plus blue or the UV-B plus UV-A light treatments the level of *CHS* expression in blue, UV-A and UV-B alone appears to be very low.

One can look at synergistic interactions as the removal of a negative

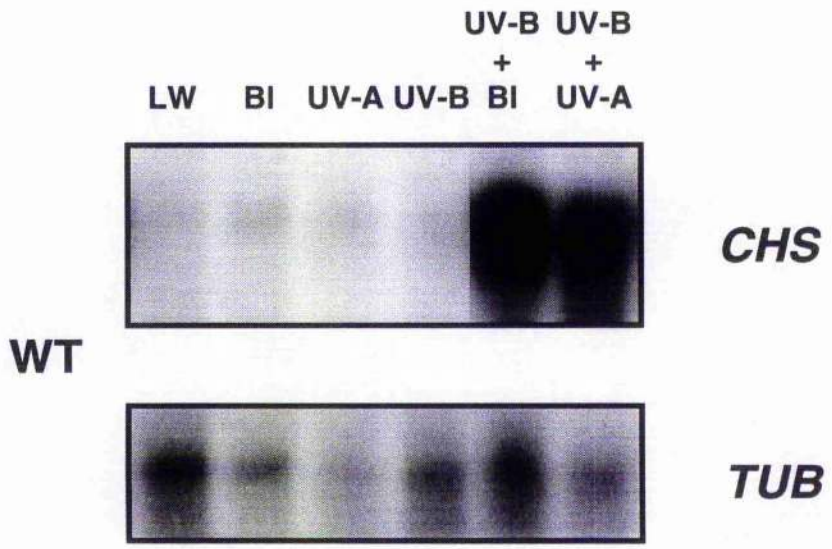


Figure 3.9.1 Wild type plants show synergism of *CHS* expression when treated with either blue and UV-B or UV-A and UV-B light

Three week old wild type plants grown in low white light were transferred for 6 hours into one of the following light conditions: blue light at 70 µmol m^{-2} s^{-1} (Bl) UV-A light at 70 µmol m^{-2} s^{-1} (UV-A), UV-B light at 3.0 µmol m^{-2} s^{-1} (UV-B) Blue and UV-B light together at the same fluence rates as above (Bl+UV-B), UV-A and UV-B light together at the same fluence rates as above (UV-A+UV-B). Control plants were harvested without additional light treatment (LW). Total RNA was isolated from leaf tissue.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

regulator of the UV-B inductive pathway. A possible explanation for the increase in the *CHS* expression seen in *icx1* after blue, UV-A or UV-B exposure was that ICX1 is a negative regulator that is removed in one of the synergistic interactions. This model is presented in Figure 3.9.2. If ICX1 was one of these negative regulators, *icx1* would be effectively always producing a synergistic level of *CHS* expression in response to illumination by a single light quality. If this was the case, *icx1* would not retain a synergistic response when exposed to either UV-B and blue or UV-B and UV-A light. If ICX1 acts specifically as the negative regulator for synergism in the UV-B/blue synergism or the UV-B/UV-A synergism, a synergistic level of *CHS* expression would be seen in the *icx1* mutant in response to one but not to the other light treatment. If ICX1 is acting as the synergism negative regulator for both synergistic pathways, the *CHS* expression in the *icx1* plants in response to both synergistic treatments would not increase above that of the single light treatment. If, for example, ICX1 was the negative regulator for the UV-B/UV-A synergistic response, *icx1* would not produce a synergistic increase in *CHS* expression on exposure to UV-B plus UV-A. If ICX1 acts upstream of the interaction of the UV-B/Blue and UV-B/UV-A synergisms, the *icx1* mutant will retain the synergistic increase in *CHS* expression after treatment with both synergistic treatments, but not show an elevated level of *CHS* expression under synergistic conditions compared to wild type.

As can be seen in Figure 3.9.3 *icx1* retains synergism with both UV-B/blue and with UV-B/UV-A light. Indeed, despite *icx1* acting downstream in the blue, UV-A and the UV-B light signal transduction pathways, comparison of *icx1* and wild type plants shows that while *icx1* retains synergism, the plants do not seem to have increased levels of *CHS* transcript above that of wild type after treatment with either UV-B/blue or UV-B/UV-A light.

CHS transcript accumulation in response to both UV-B/Blue and UV-B/UV-A is much faster than that of the individual light treatments. The increase in *CHS* transcript accumulation in both *icx1* and wild type does not

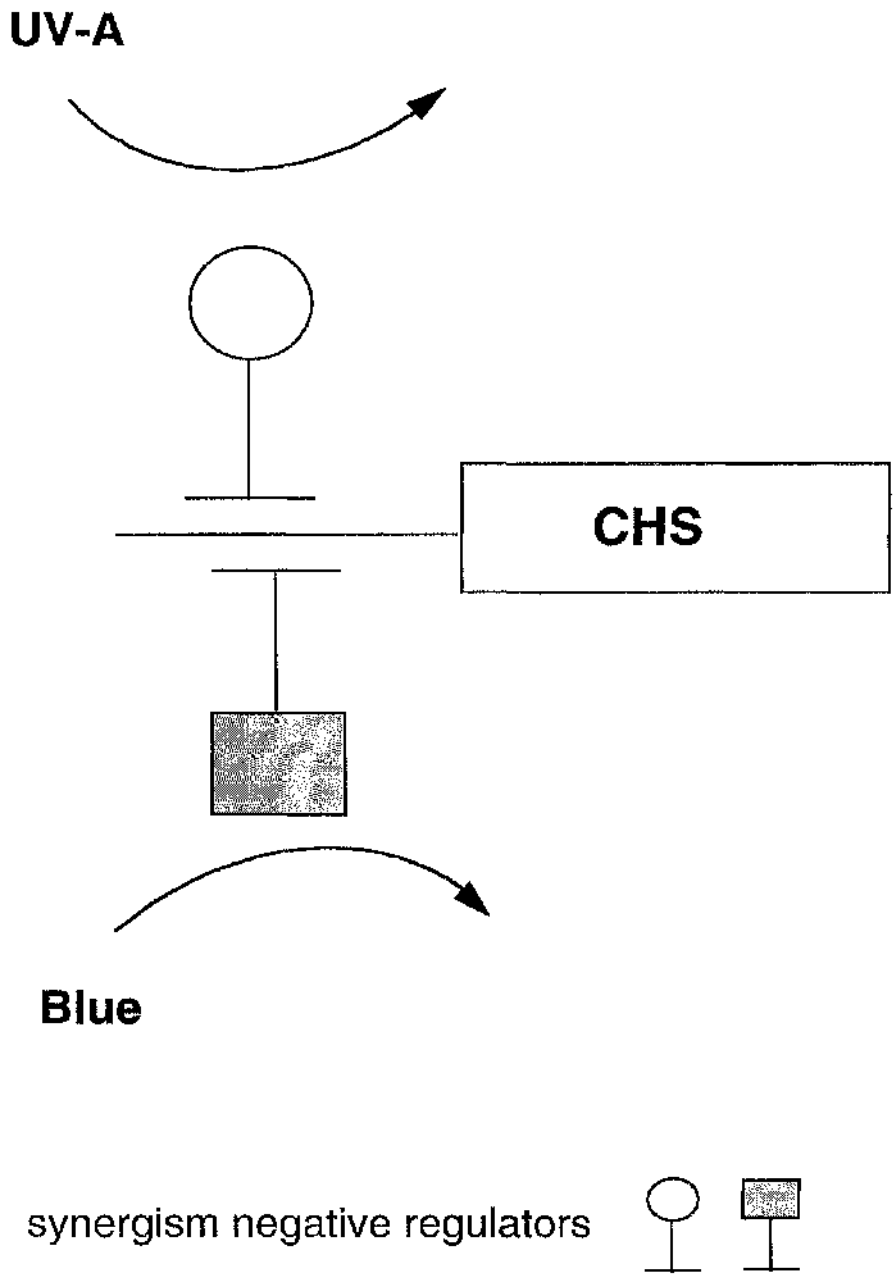


Figure 3.9.2 Model of possible synergistic interactions
Blue/UV-B or UV-A/UV-B synergism can be visualised as the removal of a synergism negative regulator by either UV-A or blue light. This removal of a negative regulator leads to the increased *CHS* gene expression.

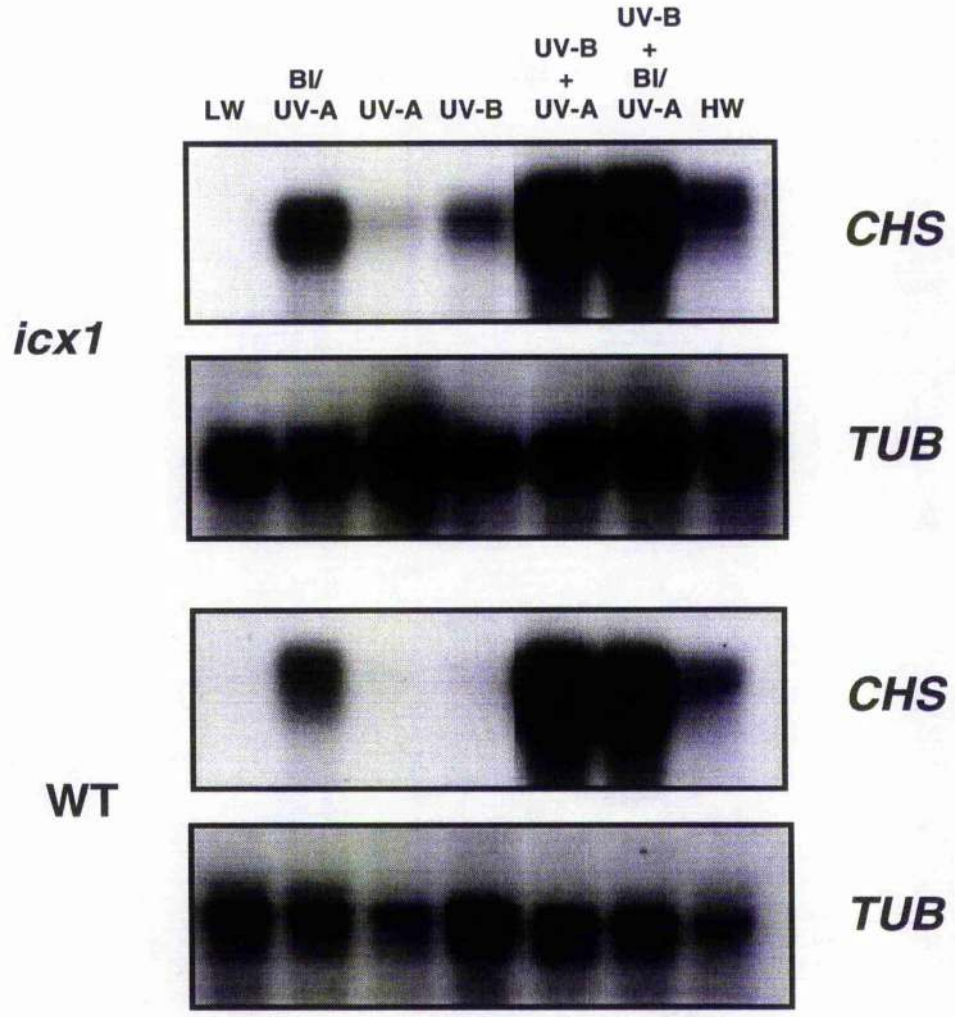


Figure 3.9.3 *icx1* retains blue/UV-B and UV-A/UV-B synergism

Three week old low white light grown wild type and *icx1* plants were transferred into 6 hours of the following light conditions. Bl/UV-A at 70 µmol $m^{-2} s^{-1}$, UV-A at 70 µmol $m^{-2} s^{-1}$ and UV-B at 3 µmol $m^{-2} s^{-1}$. HW, white light at 100 µmol $m^{-2} s^{-1}$. Combinations of light treatments retained the same fluence levels for each light treatment. Total RNA was extracted from the leaves.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

appear to be kinetically altered, however, as the increase in *CHS* transcript accumulation can be seen after two hours (see text below and Figure 3.9.4). The response is evidently fast, and alterations in kinetic accumulation of *CHS* transcripts may not have been seen at the time points initially measured. As an alteration in the relative rates of *CHS* transcript accumulation can be seen when *icx1* and wild type plants are exposed to cold, it was possible that a similar kinetic alteration was taking place upon exposure to synergistic light treatments.

To ascertain whether an alteration in the kinetics of *CHS* transcript accumulation could be seen in *icx1* plants, a further study of *icx1* and wild type plants was made. By treating plants for short lengths of time prior to the maximal induction of *CHS*, an attempt to monitor the relative rates of *CHS* mRNA accumulation in the mutant and wild type was made. Plants were treated with UV-B plus either blue or UV-A for up to two hours, samples were harvested at thirty minute intervals and total RNA was extracted and subjected to northern analysis.

Figure 3.9.4 shows that the induction of *CHS* transcripts by either UV-B/UV-A or by UV-B/blue is not kinetically altered in the *icx1* mutant from that of the wild type over the first two hours of synergistic treatment. Thus the data indicate that ICX1 is not involved in the UV-B/blue or the UV-B/UV-A synergistic responses. Therefore ICX1 cannot be one of the two negative regulators shown in the model in Figure 3.9.2.

ICX1 must then act before the point of interaction of the blue and UV-B or UV-A and UV-B light induction signals which produce the synergistic amplification of *CHS* expression. ICX1 is therefore not the negative regulator for synergism, nor is it involved in the regulation of *CHS* transcript induction in response to UV-B synergistic light treatments. Thus ICX1 can be added to Fuglevand's model of synergistic induction, see Figure 3.9.5.

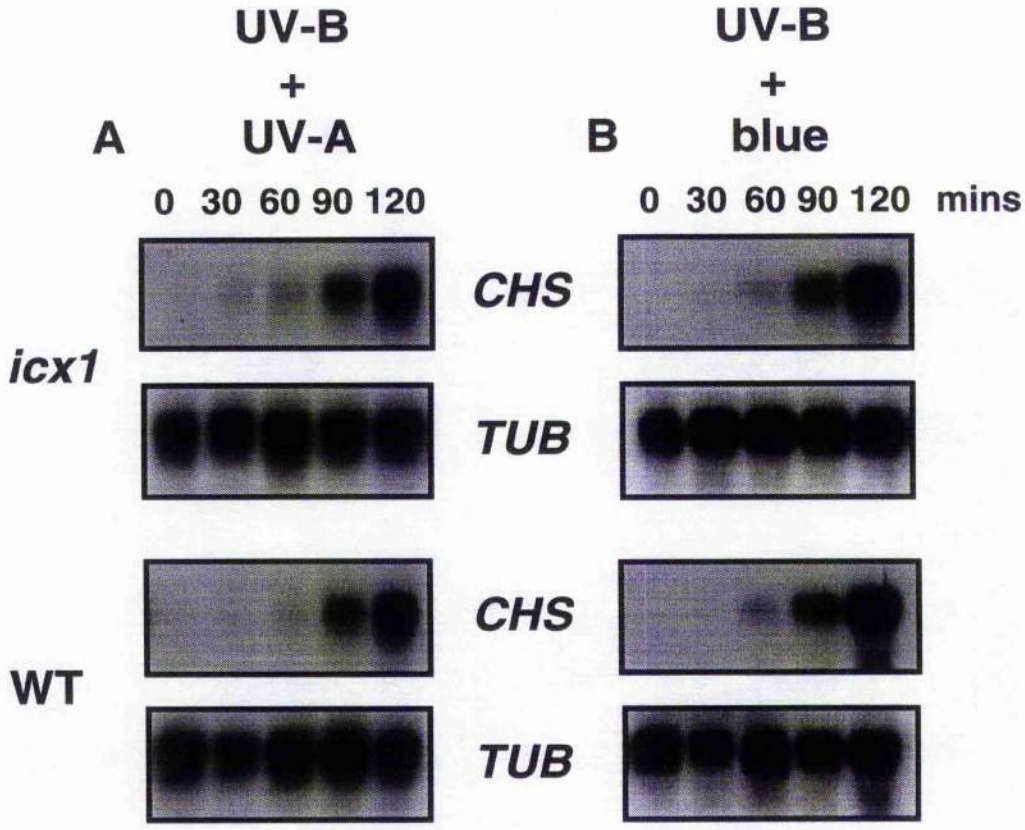


Figure 3.9.4 Kinetic accumulation of *icx1* and wild type *CHS* transcripts in Blue/UV-B and UV-A/UV-B light

This figure presents results from two separate experiments. Three week old *icx1* and wild type plants were transferred from low white light into either A) UV-B 3.1 µmol m^{-2} s^{-1} and UV-A at 82 µmol m^{-2} s^{-1} or B) UV-B at 3.3 µmol m^{-2} s^{-1} and blue light at 61 µmol m^{-2} s^{-1}. Plants were harvested after the time indicated (minutes) and total RNA was extracted from leaves.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α-tubulin (*TUB*) probe.

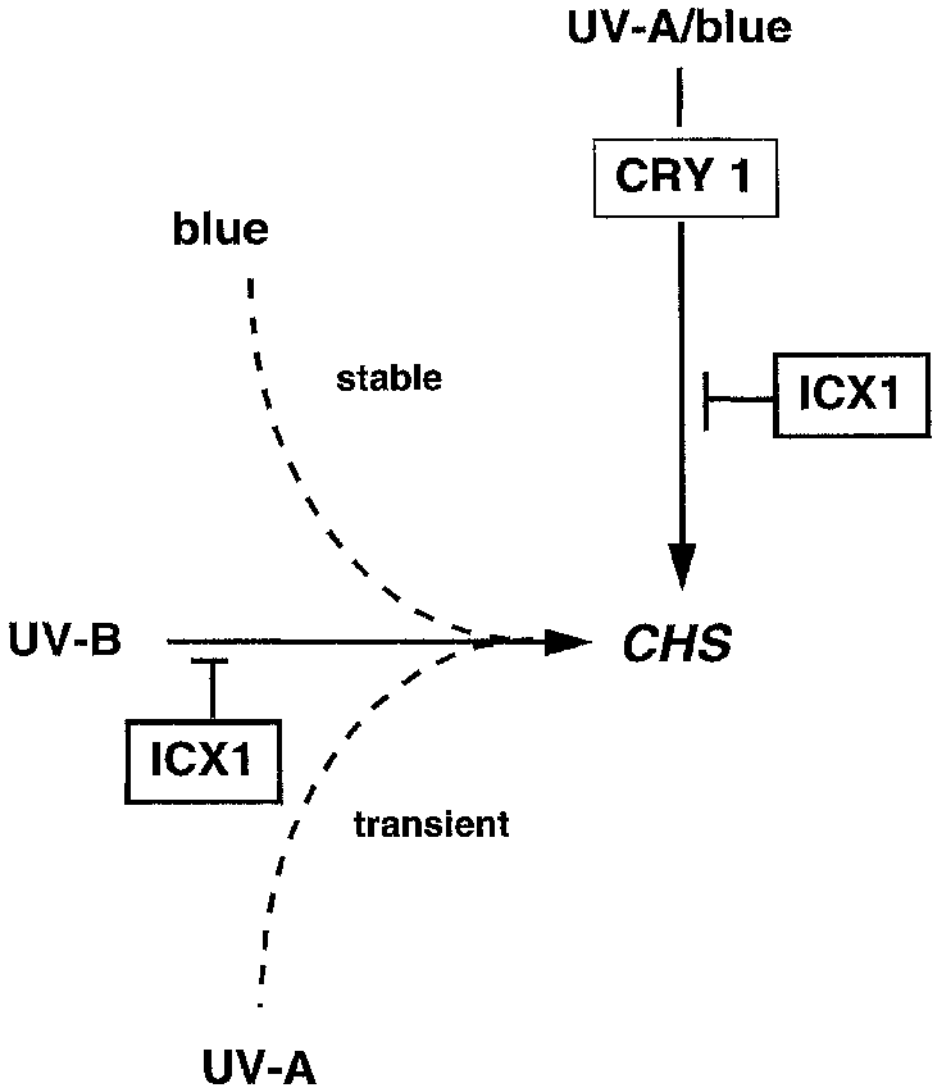


Figure 3.9.5 Model 1 of the role of ICX1 in blue/UV-B and UV-A/UV-B synergistic interactions

3.10 Discussion

As has been described here, the involvement of ICX1 in light and in some non-light mediated signalling pathways is diverse. Throughout this chapter it has been suggested that ICX1 is acting in various pathways as a negative regulator. It should be noted that it is not necessarily ICX1 itself which is negatively regulating *CHS* expression in the conditions mentioned. ICX1 may act upstream of the production of another effector which results in negative regulation of *CHS* expression in the conditions examined. How ICX1 affects the expression of *CHS* and the other flavonoid biosynthetic genes investigated here will become clearer with the cloning of ICX1. It is perhaps more likely that a number of other alterations as a result of the mutation in ICX1 cause the pleiotropic phenotype of *icx1*, rather than ICX1 acting directly to regulate *CHS*, *CFI* and *DFR* expression, sugar uptake, root hair organisation, trichome number, seed mucilage and leaf shape. However, as the nature of the interaction between ICX1 and the responses outlined will not be clarified until ICX1 is cloned, the discussion will continue to refer to ICX1 as a negative regulator, even though the regulation may be several steps removed.

3.10.1 ICX1 as a negative regulator

In three week old plants, the presence of ICX1 acts to negatively regulate responses downstream from UV-A/blue, and UV-B light receptors. It does not appear to act downstream from blue light. The blue light induced increase in *CHS* expression may be functioning through the blue light pathway which acts independently of cryptochrome 1 (Fuglevand *et al.*, 1996). That *CHS* expression is not altered between *icx1* and wild type plants in blue light indicates that either a) ICX1 does not act as a negative regulator downstream from blue light signals or b) its function as a negative regulator is altered/removed by components downstream from blue light signals. This latter possibility is countered by the observation that ICX1 is seen to act as a negative regulator downstream from blue/UV-A light signals and that CRY1 is

thought to be the main photoreceptor for both UV-A and blue light (Fuglevand *et al.*, 1996).

In mature plants, ICX1 does not act downstream from phytochromes, at least in the regulation of *CHS*, as expression of *CHS* in *icx1* is not altered from that of wild type after exposure to red or far red light. Therefore the *icx1* mutation does not alter the specificity of the *CHS* response, it results in the increase of expression under the inductive light conditions.

The developmental regulation of *CHS* expression is not affected in *icx1* plants. In the 4 day old seedlings *CHS* expression patterns in red and far red light in *icx1* and wild type plants are not altered. In the seedlings we see that where *CHS* expression is induced by far red, blue/UV-A and UV-B light in the wild type it is also induced in the *icx1* seedling and to a greater degree. In both wild type and *icx1* seedlings no red light expression was seen. As the far red light accumulation of anthocyanin appears to be mediated specifically by PHYA (Neff and Chory, 1998; Poppe *et al.*, 1998), it is likely that *CHS* expression in far red light acts downstream from PHYA. If this is the case, ICX1 is acting as a negative regulator downstream from PHYA.

The increase in *CHS* expression in *icx1* under inductive light treatments, UV-A/blue, UV-B, in both seedling and mature leaves, as well as far red in the dark grown seedlings, indicates that ICX1 plays an active role in mediating the response to inductive light treatments in both seedling and mature plants. The developmental switch from phytochrome regulation in dark grown seedlings to non phytochrome regulation in mature plants reinforces the observation that ICX1 is not restricted to acting downstream from a particular photoreceptor. This lack of photoreceptor specificity indicates that ICX1 is acting far down the signal transduction pathway from the signals, perhaps even at the *CHS* promoter itself.

icx1 shows an increase in gene expression of other enzymes involved in flavonoid biosynthesis. This increase is validated by a concomitant increase in anthocyanins (Figure 3.5.3) and flavonols (Figure 1.8.1). Whether this

upregulation of flavonoid enzymes is due to the removal of ICX1 as a negative regulator of the flavonoid gene expression or as a result of increased transcription due to feed-back or feed-forward regulation (Loake *et al.*, 1991) is unclear. However, it is clear that the regulation of those genes investigated which are not involved in flavonoid biosynthesis and do not function in the epidermis are not affected by the *icx1* mutation.

ICX1 is evidently involved in the regulation of epidermal genes which are not flavonoid genes. The visible phenotype alone indicates that ICX1 plays a role in the regulation of trichome number, epidermal cell development and root hair spacing. The light stimulated expression of an epidermal lipid transfer protein gene (*LTP*) is altered in *icx1* (Sohal, 1997). In blue and red light *icx1* has a higher level of expression of *LTP* than the wild type plants.

The role of ICX1 in mediating *CHS* expression downstream from non-light signals was also investigated. *icx1* plants transferred into 7°C displayed a faster increase in *CHS* expression than the wild type plants. A possible explanation for this is that a low temperature signalling pathway promotes an increase in *CHS* expression and that this signalling pathway is negatively regulated by ICX1.

ICX1 may affect sucrose uptake. It has been shown here (Figure 3.7.5) that in *icx1* the accumulation of sucrose is much greater than in wild type plants when both are grown on 2% sucrose. This increased level of sucrose is deleterious to the *icx1* plant, which does not survive well on 2% sucrose. Wild type plants grow well on 2% sucrose. It is not clear whether *icx1* is altered in sucrose responses as, while an increase in the level of expression in high white light is seen, it is similar in magnitude to the increase seen in soil grown plants. In the absence of sucrose the level of *CHS* expression in both wild type and *icx1* seedlings is much reduced (Figure 3.7.3). If sugar is required for *CHS* expression in *Arabidopsis* plants, as suggested by the work of Valentine (1998) in cell culture, the only sugar source available to the seedlings grown on –S would be that from photosynthesis. As the plants were transferred from darkness into

either low or high white light for 6 hours prior to harvesting, the low level of *CHS* expression may be due to a limiting amount of sucrose in the plants. Figure 3.7.5 indicates that in both *icx1* and wild type plants grown in the dark and transferred into low white light for 6 hours, a low level of sugar is seen. As the sugar level is low in *icx1* and WT it may be that sugar is limiting the level of *CHS* expression. It should be noted that in low white light –S *icx1* and wild type plants have almost identical levels of sugar content, indicating that *icx1* plants have the same photosynthetic capability to produce sugars as the wild type plants. The differences in sugar content seen in +S are therefore likely to be due to differences in sucrose uptake. The *icx1* mutant has altered root and root hair development, this may have an effect on the number or activity of sucrose transporters.

With respect to where ICX1 functions in the light signal transduction pathway, double mutant studies indicate that ICX1 acts downstream from cryptochrome 1. In the absence of CRY1 there is a reduction in blue/UV-A light mediated *CHS* expression in the *icx1* mutant, as in the *hy4* parent. This is consistent with the data indicating that ICX1 acts downstream of several photoreceptors. It is further noted that *CHS* expression is not increased in *icx1* as a result of a greater accumulation of cryptochrome 1 transcripts (Figure 3.4.1). Transient expression studies reported by Valentine (1998) indicate that increased *CRY1* expression results in increased *CHS* expression upon inductive light exposure. The increase in *CHS* expression seen in the *icx1* mutants is not due to an increase in CRY1 expression and requires CRY1 for the bulk of the *CHS* expression seen in UV-A light. Therefore ICX1 is acting downstream from CRY1 in the blue/UV-A signal transduction pathway.

Investigation of the interaction between ICX1 and HY5 remains inconclusive. HY5 is required for *CHS* expression in white light (Ang *et al.*, 1998), probably mediated through HY5 binding to the *CHS* promoter via the G-box motif (Ang *et al.*, 1998; Oyama *et al.*, 1997). From Section 3.8.2 it can be seen that HY5 is also required for *CHS* expression in UV-A and UV-B light. Removal

of ICX1 does not alter this HY5 requirement. HY5 alone is insufficient for *CHS* expression, it requires interaction with transcriptionally active partner(s) (Ang *et al.*, 1998). As HY5 is apparently required for *CHS* expression and binds the *CHS* promoter, it is likely that ICX1 either also acts at the point of interaction with the promoter, or that it acts upstream of HY5 or one of the transcription partners.

In blue plus UV-B and UV-A plus UV-B synergism no difference is seen between the levels of gene expression in wild type and *icx1* plants. This lack of negative regulation by ICX1 could be as a result of two possibilities. Either ICX1 is not involved in synergism, perhaps functioning upstream from the point of interaction of synergism, or ICX1 is a negative regulator which is removed in both blue plus UV-B and UV-A plus UV-B synergistic conditions. In the latter possibility, the removal of ICX1 negative regulation may result (in part) in the synergistic increase in *CHS* expression seen. If this is the case, it is unlikely that ICX1 is the only regulator functioning in blue plus UV-B and UV-A plus UV-B synergism. The levels of gene expression seen under synergistic conditions are much greater than *CHS* transcript accumulation seen in the *icx1* mutant after either UV-B or blue or UV-A light treatments alone. If the synergism model presented in Section 3.9.2 of negative regulators being removed by UV-A or blue light to result in a synergistic effect is extrapolated, it is possible that ICX1 is an additional negative regulator which is removed regardless of the combination of synergistic treatments. This model is shown in Figure 3.10.1. This removal of at least two negative regulators, ICX1 and the negative regulator connected with either UV-A or blue light, depending on the treatment, could create the much larger increase in *CHS* expression seen under UV-B synergistic conditions. This would also explain the fact that no difference is seen between wild type and *icx1* under UV-B synergistic conditions.

In blue light alone it was noted that *CHS* expression in *icx1* was not consistently greater than in the wild type plants. As can be seen in Figure 3.8.1 (see also Fuglevand *et al.*, 1996) in the *hy4* mutants, induction of *CHS* expression

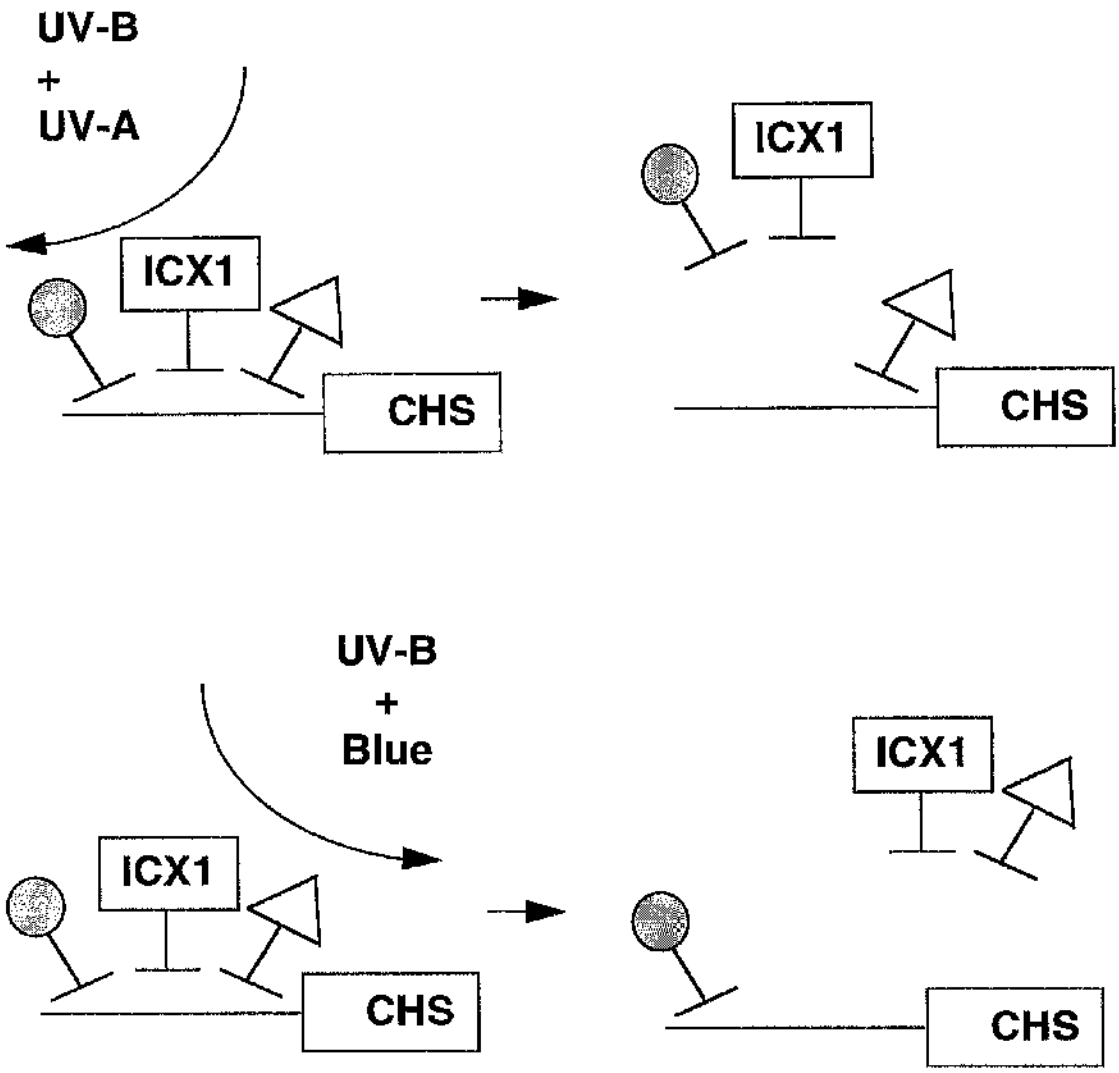


Figure 3.10.1 Model 2 of the role of ICX1 in blue/UV-B and UV-A/UV-B synergism

ICX1 may be acting as a negative regulator in both synergistic responses. It may be removed under conditions of either UV-A plus UV-B or blue plus UV-B light.

could still be seen in blue light. This induction is probably mediated by another cryptochrome or by phytochrome, or by a combination of the types of photoreceptors. It is possible that ICX1 may also be removed in response to blue light alone as no consistent difference in the level of transcript accumulation is seen between wild type and *icx1* plants.

Chapter 4: Phytochromes and UV Light Detection

4.1 Introduction

As previously described in Section 1.3 there are many documented interactions between photoreceptors mediating various downstream responses. The availability of mutants lacking one or more of the photoreceptors allows interaction between members of different photoreceptor families to be more easily studied. It appears that the input from various photoreceptors is variable depending on the specific response investigated. Many responses primarily mediated by a single light quality have been shown to involve more than one photoreceptor. For example, blue light phototropism involves NPH1 and the two cryptochrome photoreceptors (Ahmad *et al.*, 1998c; Christie *et al.*, 1998).

It has long been known that there is an interaction between red and blue light and this interaction has been termed co-action. The effect of a red light pretreatment enhances blue light mediated phototropic curvature in *Arabidopsis* hypocotyls. This red light effect is primarily mediated by phytochrome A (Parks *et al.*, 1996). Cryptochrome 1 and 2 have also been shown to be involved in mediating the phototropic response, though with a high level of redundancy between the two cryptochromes, CRY1 and CRY2 (Ahmad *et al.*, 1998a; Christie *et al.*, 1998).

Red light treatment preceding blue light also affects anthocyanin accumulation, increasing the anthocyanin accumulation from that obtained by blue light alone (Ahmad and Cashmore, 1997; Mancinelli *et al.*, 1991). The effect of a red light pretreatment on anthocyanin accumulation is well documented (Mancinelli *et al.*, 1991, and references therein) and as such we would expect to see an increase in gene expression leading to anthocyanin production, upon inductive red/blue treatment. Phytochrome A and B double mutants are impaired in both CRY1 mediated hypocotyl elongation and anthocyanin accumulation (Ahmad and Cashmore, 1997; Casal and Bocalandro, 1995).

The interaction between different light signalling pathways indicates

there is cross talk between the different pathways downstream from the photoreceptors or at the photoreceptors themselves. Indications that phytochrome and cryptochrome functionally interact in vitro has been provided by the work of Ahmad *et al.* (1998b). The yeast two hybrid system indicates that cryptochrome 1 and phytochrome A interact. Further to this, phosphorylation of cryptochrome 1 does not occur in vitro with cryptochrome alone, however, in the presence of phytochrome A, phosphorylation of CRY1 is observed. In vivo, phosphorylation of CRY1 was observed and was shown to increase in red light (Ahmad *et al.*, 1998b).

These observations lead to a model of the interaction of phytochrome and cryptochrome where red light causes an activation of phytochrome. Blue light illumination causes partial activation of cryptochrome, this partial activation can then be increased by interaction with the active form of phytochrome. The light activated forms of phytochrome and cryptochrome must be stable as both red light followed by blue and blue light followed by red, results in an increase in downstream responses from cryptochrome (Ahmad *et al.*, 1998b).

That red light pretreatment of *Arabidopsis* plants can produce an increase in anthocyanin accumulation indicates the involvement of phytochrome(s) in regulating *CHS* expression in blue light (Casal and Bocalandro, 1995). That anthocyanin accumulation is reduced in *phyA phyB* mutants reinforces this (Ahmad and Cashmore, 1997). This regulation may be a result of the increased activation of CRY1 by phytochrome. If this is the case it is likely that phytochromes are also involved in the mediation of responses downstream from UV-A as cryptochrome absorbs in the UV-A region of the spectrum. What of phytochrome interactions with UV-B photoreceptors? There is evidence that phytochrome mediates photomorphogenic changes in response to low UV-B fluence rates (Kim *et al.*, 1998). In this chapter the involvement of phytochrome in UV light responses is examined.

To further investigate the interactions between downstream light

signalling intermediaries, single and double phytochrome A and B mutants were utilised. The increase in anthocyanin accumulation in response to red light pretreatments must result in an increase in *CHS* expression, or in a decrease in turnover of the CHS protein.

Initially, the level of *CHS* expression in UV-A was compared between wild type, a phytochrome A null mutant, a phytochrome B null mutant (*hy3*) and the double null phytochrome A, phytochrome B mutant (*phyAphyB*). In the previous chapter the *CHS* expression produced in mature wild type plants was characterised in the experimental conditions used here (Section 3.2). To further investigate this in mature plants, the phytochrome null mutants were utilised.

4.2 Involvement of Phytochrome in Blue Light Regulation

It has been noted that red and far red light do not increase *CHS* expression in mature *Arabidopsis* seedlings (Section 3.2). This lack of expression in red and far red indicates that phytochrome activation alone is not sufficient for *CHS* expression in mature plants. Far red illumination results in *CHS* expression in dark grown seedlings (Kaiser *et al.*, 1995, Section 3.3). In developing seedlings, blue light produces an increase in anthocyanin accumulation. This anthocyanin accumulation is enhanced by a red light treatment prior to or at the same time as a blue light exposure (Ahmad and Cashmore, 1997; Mancinelli *et al.*, 1991). This indicates that phytochrome plays a role (albeit indirect) in the regulation of *CHS* expression. However, Batschauer *et al.* (1996) reported that in two day old dark grown seedlings containing either a full length *CHS1* promoter-GUS fusion or a Unit 1 construct from white mustard, no difference in GUS expression was seen between wild type plants and *phyA*, *phyB* single mutants, or *phyA phyB* double mutant plants exposed to blue or UV-A light. The conclusion was of independence between phytochrome and cryptochrome.

At least some of the studies mentioned above indicate that in blue light phytochrome is involved in anthocyanin accumulation. There is no information

in the literature indicating whether phytochrome null mutants are altered in UV-A and UV-B light regulation of *CHS* expression measured directly.

4.3 Phytochrome Regulation of *CHS* Expression in UV-A Light

Phytochromes absorb light in the UV-A region of the spectrum and may affect *CHS* expression in UV-A light. Also, UV-A is detected via CRY1. Plants absorbing blue light via CRY1 are known to have reduced anthocyanin accumulation in the absence of PHYA and PHYB (Ahmad and Cashmore, 1997). To test whether phytochrome was involved in mediating *CHS* expression in UV-A light, expression levels of *CHS* in phytochrome A and phytochrome B single mutants were compared with the expression levels of wild type plants.

Three week old, low white light grown wild type and *phyA* single mutant plants were transferred for six hours into UV-A light of 87 μmol m^{-2} s^{-1} (Figure 4.3.1A) and as shown in Figure 4.3.1B, wild type and *phyB* single mutants were transferred for six hours into UV-A light of 70 μmol m^{-2} s^{-1}, the level of *CHS* expression in response to the UV-A light treatments was determined by northern analysis for each. The level of *CHS* expression obtained in the *phyA* mutant plants was very similar to that obtained in the wild type plants treated under the same light conditions. The *phyB* mutants consistently displayed a reduced level of *CHS* expression compared to that of wild type when exposed to six hours of UV-A light.

The phytochrome A, phytochrome B double mutant, *phyAphyB*, provided the opportunity to test whether there was some element of overlapping roles between phytochrome A and phytochrome B in the regulation of *CHS* expression in UV-A light.

As can be seen in Figure 4.3.2 wild type plants, *phyA phyB* double mutant, *phyA* and *phyB* single mutants were transferred into UV-A light of 80 μmol m^{-2} s^{-1} for six hours. As before, there is no real difference between the expression levels of *phyA* and wild type. The expression in *phyB* is much reduced from that of wild type, as is the *CHS* expression in the *phyA phyB*

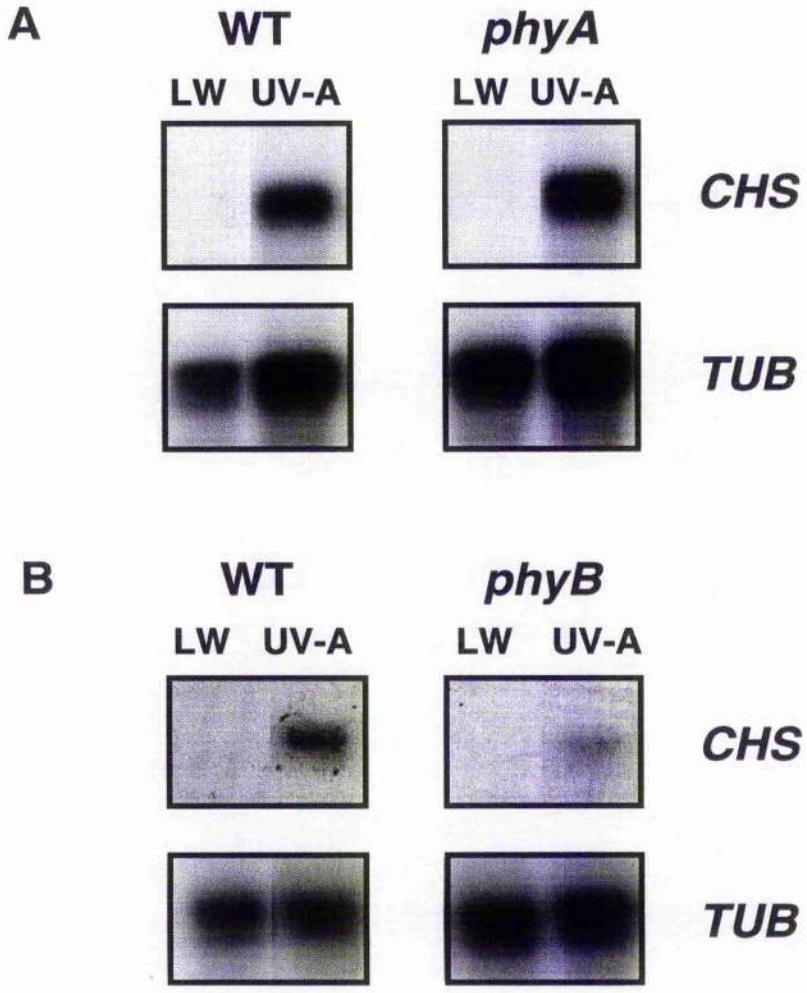


Figure 4.3.1 Phytochrome is involved in UV-A light mediated *CHS* expression.

Three week old low white light grown plants were transferred into UV-A light for six hours (UV-A). A) wild type and *phyA* plants were exposed to UV-A light at 87 µmol m^{-2} s^{-1}. B) wild type and *phyB* plants were exposed to UV-A light at 70 µmol m^{-2} s^{-1}. In both A) and B) control plants remained untreated (LW). RNA was isolated from leaf tissue.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe.

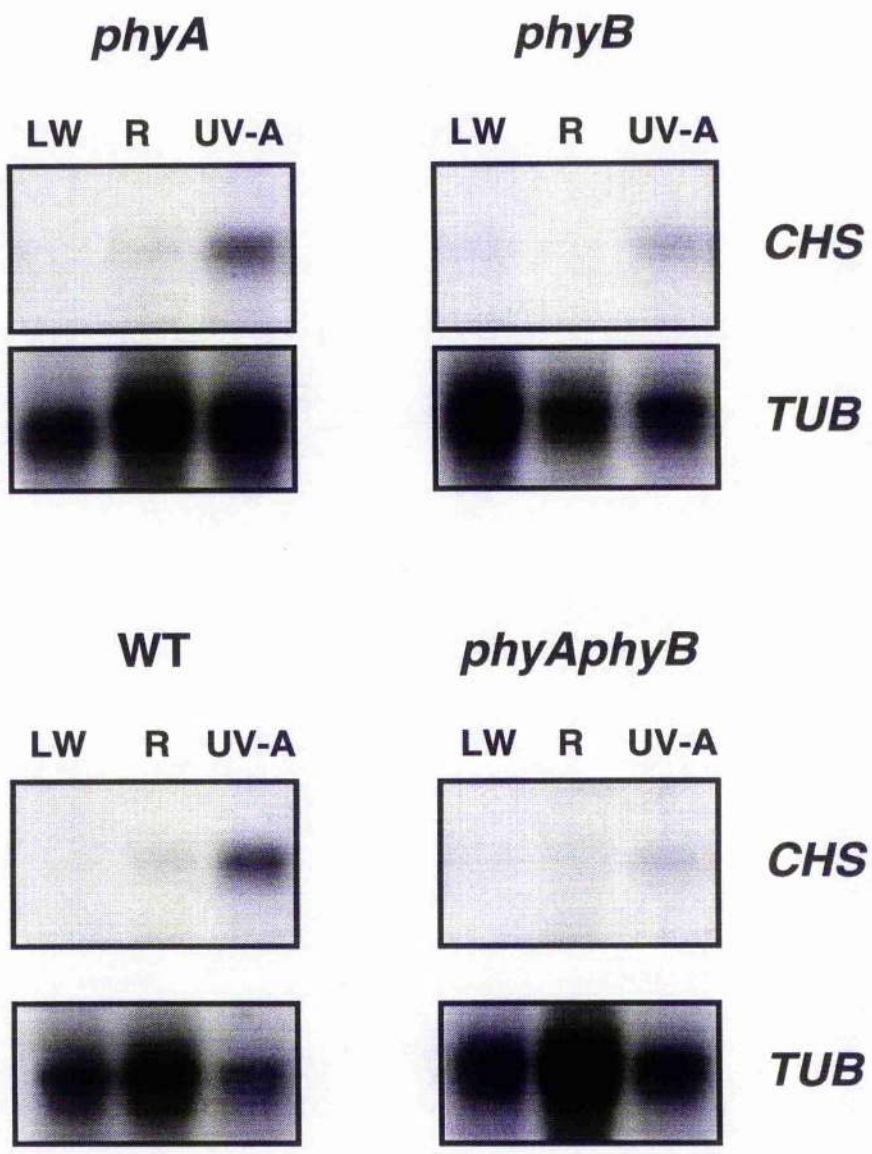


Figure 4.3.2 Phytochrome is involved in *CHS* expression in UV-A light

Three week old low white light grown plants were transferred into either red light at 60 µmol m^{-2} s^{-1} (R) or UV-A light at 80 µmol m^{-2} s^{-1} (UV-A) for six hours. LW indicates untreated control plants. After the six hour treatment the plant leaves were harvested and RNA isolated. Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS)* probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe.

double mutant. The *phyA phyB* double mutant has a slightly lower level of *CHS* expression in UV-A light than the *phyB* single mutant indicating that PHYA does play a small role in regulating the expression of *CHS* in three week old seedlings. The absence of PHYB leads to a reduction in *CHS* expression in response to UV-A light.

4.4 Phytochrome Regulation of *CHS* Expression in UV-B light

A recent study by Kim *et al.* (1998) has indicated that phytochromes may be involved in mediating photomorphogenic responses to low fluence rates of UV-B light in *Arabidopsis* seedlings. The low fluence rate used by Kim *et al.* was 0.22 μmol m^{-2} s^{-1}; the high fluence rate used in their study was 2.1 μmol m^{-2} s^{-1}, which caused damage to five day old seedlings. Kim *et al.* found that neither the *phyA* or the *phyB* single mutant was altered in hypocotyl inhibition in 0.22 μmol m^{-2} s^{-1} UV-B light, whereas the *phyA phyB* double mutant showed a reduction in hypocotyl extension inhibition. They concluded that either PHYA or PHYB was involved in this low fluence rate mediated response.

To investigate whether there is involvement of phytochromes in the *CHS* transcript induction by UV-B, the *Arabidopsis* phytochrome A null mutant *phyA* and the phytochrome B null mutant *phyB* were used, as was the double null *phyAphyB* mutant. The expression of *CHS* transcripts was compared between the mutants and the wild type plants after UV-B illumination.

Wild type, *phyA* and *phyB* plants were grown for three weeks under levels of low white light which did not induce *CHS* expression. These plants were then illuminated for four hours with 3.3 μmol m^{-2} s^{-1} of UV-B light. As can be seen in Figure 4.4.1 the *phyB* plants consistently produced a higher level of *CHS* transcripts than either the wild type or the *phyA* mutant in response to UV-B treatment. This would indicate that phytochrome B plays a role in mediating the induction of *CHS* transcript levels in response to UV-B irradiation, and that its role is one of negative regulation.

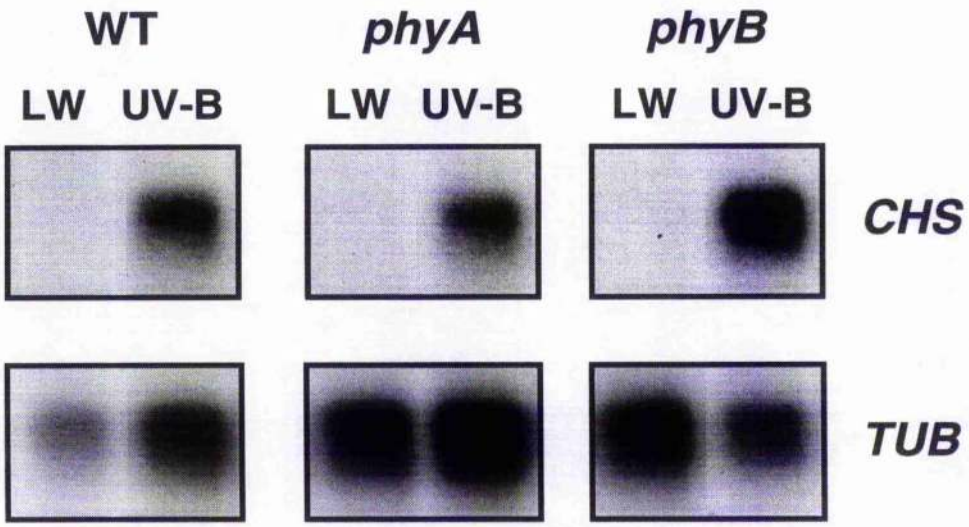


Figure 4.4.1 *CHS* induction by UV-B light in the *phyA* and *phyB* mutants compared to that of wild type.

Three week old low white light grown plants were irradiated with 3.3 μmol $m^{-2}s^{-1}$ UV-B light for 4 hours. Leaf tissue was harvested and total RNA was isolated.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe.

In order to ensure that the increased level of *CHS* expression seen in the *phyB* mutant was not a result of over compensation of PHYA, the same light treatment (3.3 μmol m^{-2} s^{-1} for four hours) was given to the *phyAphyB* double mutant, the single mutants, and wild type. As can be seen in Figure 4.4.2 the double mutant retained the increase in *CHS* expression after exposure to UV-B light (the apparent lack of induction of *CHS* by UV-B is a result of the short exposure of the autoradiograph to film, the higher level of expression in *phyB* and *phyA phyB* can be seen). This would imply that either a) phytochrome B is a negative regulator of *CHS* expression in response to UV-B light or b) that another of the phytochromes is involved in positive regulation and its role is enhanced by the removal of the phytochrome B, perhaps due to an increase in expression in the absence of PHYB.

The time point chosen for examination of the UV-B light induction of *CHS* was the one seen in wild type to have the highest level of expression. This four hour time point has given us an indication that there are alterations between the *phyB* mutant and the wild type response. A time course comparing the *CHS* expression of wild type plants with *phyA*, *phyB* and the *phyAphyB* double mutant under the same conditions gives more detail about the alteration of expression. Figure 4.4.3 shows a representative autoradiograph of the time courses made with these mutants in UV-B light. Leaf tissue was harvested after 0, 2, 4, 6 and 24 hours of UV-B treatment at 3.3 μmol m^{-2} s^{-1}

CHS transcript expression of the *phyB* and *phyAphyB* plants remains higher than that of wild type over the length of the time course. In particular the level of *CHS* expression after a 6 hour illumination with UV-B light consistently resulted in a higher level of *CHS* expression in the *phyB* and the *phyA phyB* mutants. Whether it follows that there is an increase in anthocyanin accumulation in these mutants has not been investigated. It is perhaps more likely that the increase in flavonoids downstream from *CHS* occurs mainly in kaempferol and other UV absorbing flavonoids. Whether there is post

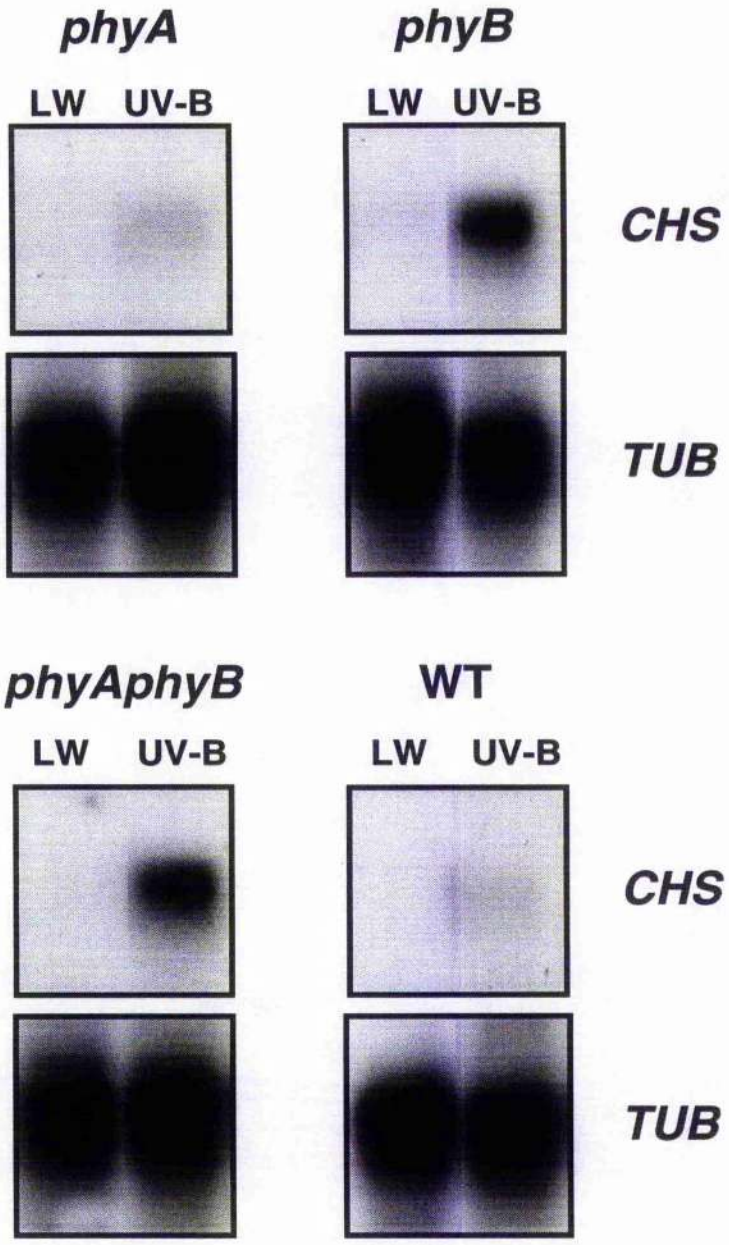


Figure 4.4.2 *phyB* and *phyAphyB* are altered in UV-B light mediated *CHS* expression

Three week old *phyAphyB* double mutants, *phyA* and *phyB* single mutants and wild type plants were transferred into 3.3 µmol $m^{-2} s^{-1}$ UV-B light (UV-B) for four hours. Control plants were untreated (LW). Leaves were then harvested and RNA isolated.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe. The low level of *CHS* expression seen in the WT after UV-B exposure is due to the short duration of exposure of the film to the probed filter.

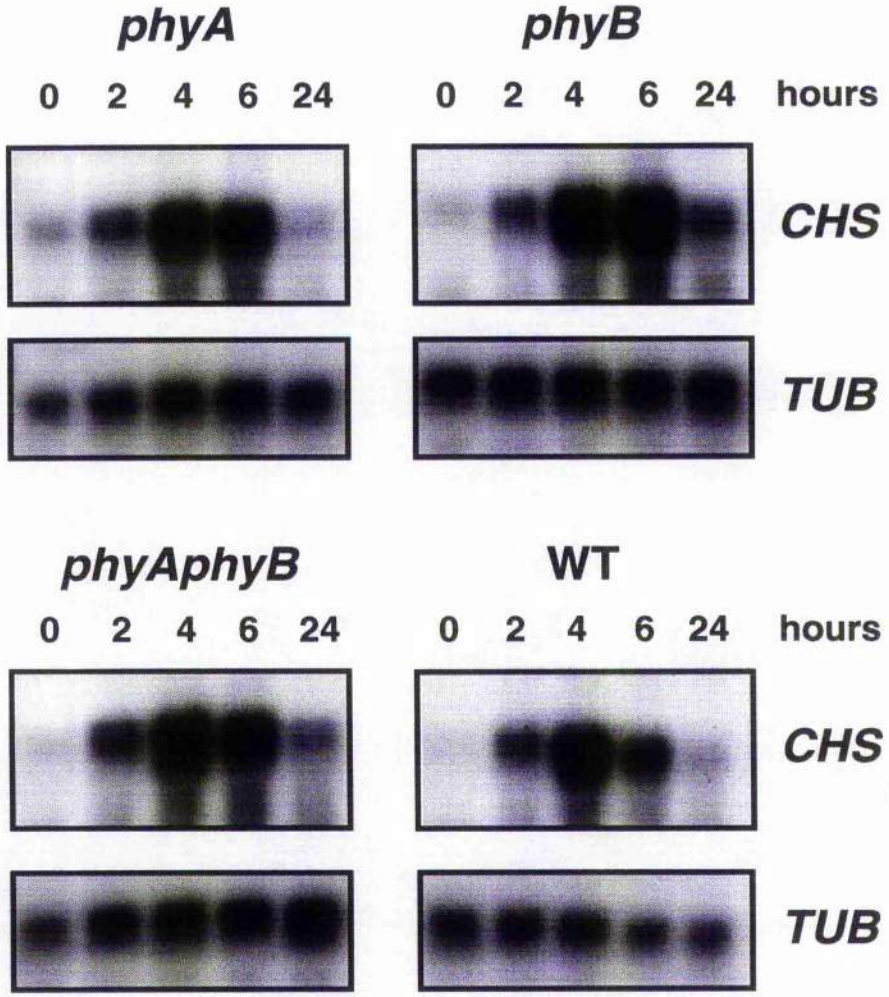


Figure 4.4.3 *CHS* expression in *phyA*, *phyB* single and *phyAphyB* double mutants in UV-B light

Three week old *phyA*, *phyB* single and *phyAphyB* double mutants and wild type plants were transferred from low white light (20 µmol m^{-2} s^{-1}) into 3.3 µmol m^{-2} s^{-1} UV-B light. Plants were harvested after the noted times and RNA isolated from leaf tissue.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe.

transcriptional regulation of the *CHS* transcripts or not, it remains that PHYB is involved in the repression of *CHS* transcript induction under UV-B light.

4.5 Interaction of Red and UV/Blue Light in *CHS* Expression

4.5.1 Interaction of Red and Blue Light in *CHS* Expression

Anthocyanin accumulation increases above that produced by blue light alone in both monocot and dicot species if the plants are first or also exposed to a red light pretreatment (Ahmad and Cashmore, 1997; Mancinelli *et al.*, 1991). As mentioned in the introduction to this chapter (Section 4.1) cryptochrome and phytochrome have been shown to interact, both by studies of responses (anthocyanin accumulation Ahmad and Cashmore, 1997; hypocotyl length inhibition, Casal and Bocalandro, 1995) and by in vitro studies of interaction (Ahmad *et al.*, 1998b).

The increase in *CHS* expression which results in increased anthocyanin accumulation can be seen as a result of a red light pretreatment. Figure 4.5.1 is a northern analysis of three week old plants transferred from low white light into either red light at 50 µmol m^{-2} s^{-1} for six hours, into blue/UV-A light at 75 µmol m^{-2} s^{-1} for six hours or subjected to a red light pretreatment of one hour prior to a six hour blue/UV-A treatment as described above. It can be seen in the system utilised here, a one hour red light treatment followed by six hours of blue/UV-A light results in an increase in *CHS* expression greater than that seen in blue/UV-A light alone.

These red and blue light interactions can be extended to UV-A light using *CHS* expression to ascertain whether the red light induced increase in *CHS* expression is seen in UV-A light alone. UV-A light regulation of *CHS* appears to be entirely mediated by CRY1 (Fuglevand *et al.*, 1996). An effect of red light pretreatment and UV-A light would be expected if the blue and red light interaction is mediated through CRY1.

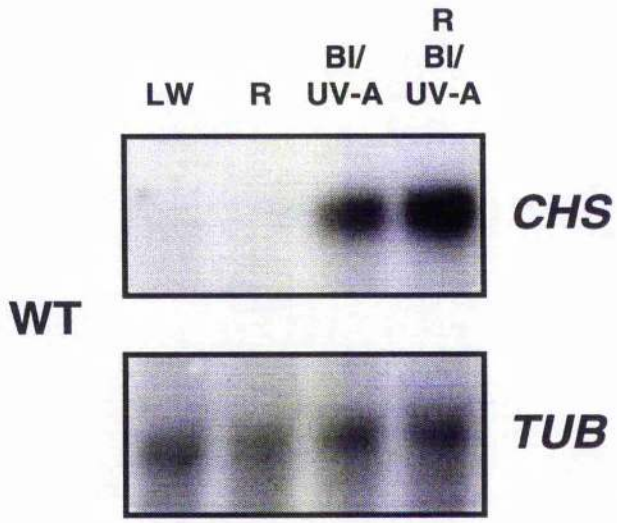


Figure 4.5.1 Red light pretreatment increases blue/UV-A light induced *CHS* expression

Three week old wild type plants were transferred into red at 50 µmol m^{-2} s^{-1} or blue/UV-A light at 75 µmol m^{-2} s^{-1} for six hours (Bl/UV-A) or were first transferred into red light for one hour at 50 µmol m^{-2} s^{-1} prior to a six hour blue treatment as described above (R Bl/UV-A). Untreated plants remained in low white light (LW). After treatment total RNA was extracted from the leaf tissue.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe.

4.5.2 Interaction of Red and UV-A Light in *CHS* Expression

The initial blue and red light interaction experiments were conducted on developing seedlings. Here, gene expression upstream from the anthocyanin accumulation in mature *Arabidopsis* plants was investigated. In Sections 4.3 and 4.4, it was concluded that phytochrome has a role in regulating the *CHS* expression in response to both UV-A and UV-B light. Whether this phytochrome regulation results in a red light enhancement of the UV-A response is explored here. The involvement of phytochromes A and B in this increase is also established.

In the experimental conditions used it was observed that when three week old plants grown in low white light were given a 1 hour red pretreatment at 50 μmol m^{-2} s^{-1} before a 6 hour UV-A treatment (80 μmol m^{-2} s^{-1}) they showed a greater accumulation of *CHS* transcripts than the plants which had only been exposed to 6 hours UV-A at the same fluence rate. Plants which had been exposed for 6 hours to red light showed no *CHS* transcript accumulation (Figure 4.5.2). The red/UV-A interaction is a synergistic one. The *CHS* transcript accumulation in the combined light treatments is greater than the sum of the two treatments. The red light level of *CHS* mRNA accumulation is negligible so the additive level of expression of the two treatments would be the same as the UV-A *CHS* expression level.

As seen in Section 3.2, three week old plants treated with red light alone show no increase in *CHS* transcript levels, even after extended light treatments. Therefore the increase seen in *CHS* expression with a red light pretreatment is not due directly to phytochrome absorption. It is perhaps due to the activation of phytochrome which then activates cryptochrome or a downstream signalling component, allowing for a greater response when UV-A light is detected by cryptochrome.

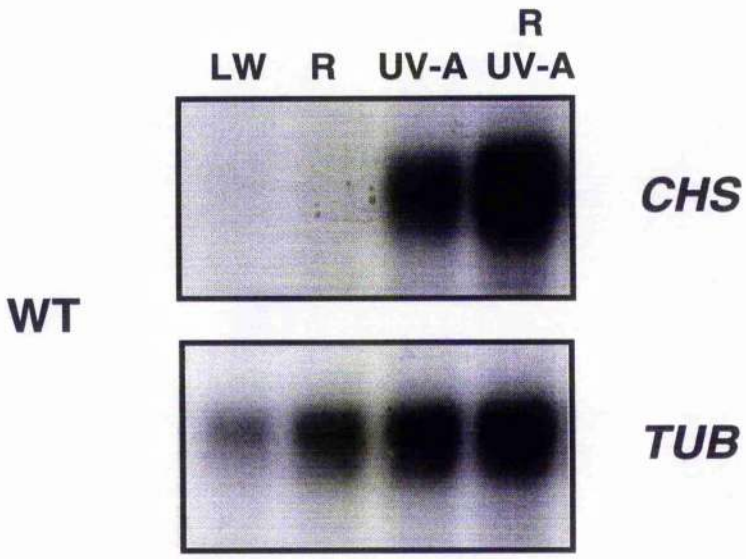


Figure 4.5.2 Red light pretreatment increases *CHS* expression in plants exposed to UV-A light

Three week old low white light grown plants were exposed to either red light at 100 µmol m^{-2} s^{-1} (R) UV-A light at 80 µmol m^{-2} s^{-1} (UV-A) for six hours or to one hour of red light prior to transfer into UV-A for six hours, light fluence rates as above (R UV-A). Total RNA was isolated from leaves.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe.

4.5.3 Red Light Interaction with UV-B Light in *CHS* Expression

Interactions with red light have been seen in both blue/UV-A and UV-A light. This may be explained by an interaction between phytochrome and cryptochrome. To extend the investigation of interactions of phytochrome to the UV-B light photoreceptor, red light pretreatments were given prior to UV-B light exposures. Unfortunately, the effect of a red light pretreatment followed by four hours of UV-B light was inconclusive. Eight repeats resulted in four analyses yielding an increase in *CHS* expression after red light pretreatment, three showing no increase and one showing a slight decrease in *CHS* expression after red light pretreatment. As no distinct trend could be seen, the more reliable red/UV-A interaction was further investigated.

4.5.4 Magnitude of Increase of *CHS* Expression after Red Light Treatment

To ascertain the relative increase in *CHS* expression obtained by a red light pretreatment followed by UV-A light, the expression level of plants exposed to red light, to UV-A light, and to red followed by UV-A, were compared to plants treated with the synergistic light treatment of UV-B and blue light (see Section 3.9). As can be seen in Figure 4.5.3 the *CHS* expression level of plants treated with blue light at 100 µmol m^{-2} s^{-1} and UV-B at 3.3 µmol m^{-2} s^{-1} is much greater than that of the plants exposed to one hour of 75 µmol m^{-2} s^{-1} red light followed by 75 µmol m^{-2} s^{-1} of UV-A light. This shows that the synergistic level of increase in *CHS* expression in red followed by UV-A light is much lower than that of the blue/UV-B synergistic interaction.

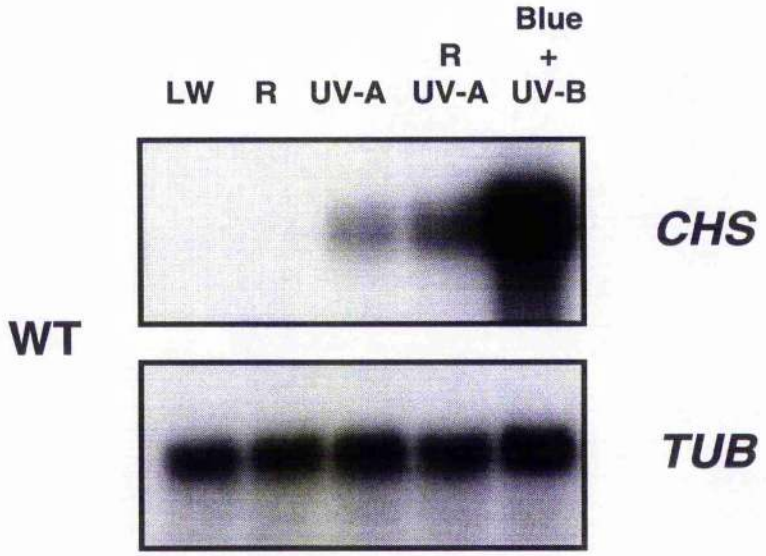


Figure 4.5.3 Red pretreatment followed by UV-A does not produce as large a *CHS* expression as blue+UV-B light

Three week old wild type plants were illuminated for 6 hours with red light (R) at 75 µmol $m^{-2} s^{-1}$, UV-A light at 75 µmol $m^{-2} s^{-1}$ (UV-A), UV-A after one hour of red light treatment (R UV-A) or six hours of blue (100 µmol $m^{-2} s^{-1}$) and UV-B light (at 3.3 µmol $m^{-2} s^{-1}$) together (Bl+UV-B). Leaf tissue was harvested and RNA isolated.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe.

4.6 What is Mediating the Red/UV-A Synergistic Response?

PHYA and CRY1 show a level of interaction in a number of responses. The circadian oscillation of a catalase CAT3 in wild type *Arabidopsis* normally damps to a high level of expression in extended dark conditions, this damping does not occur in mutants lacking either CRY1 or PHYA (Zhong *et al.*, 1997). Ahmad and Cashmore (1997) reported that CRY1 has a functional dependence on both PHYA and PHYB and that the activity of the CRY1 photoreceptor can be manipulated by altering the levels of active phytochrome by red and far red light pulses. There was also a decrease in anthocyanin accumulation seen in phytochrome deficient double mutants, *phyA phyB*, after blue light illumination (Ahmad and Cashmore, 1997), though no difference was seen in *CHS* expression in these plants from that of the wild type as measured by ß-glucuronidase (Batschauer *et al.*, 1996). In UV-A light we see a decrease in UV-A light mediated *CHS* expression in *phyB* mutants (Section 4.4).

As cryptochrome absorbs in the blue/UV-A spectrum, it is likely to be involved in the perception of UV-A in this response. Phytochromes absorb red and far red light and therefore one or more of the phytochromes could be involved in the perception of the red light. The involvement of cryptochrome and phytochromes can be tested using plants defective in the biosynthesis of these light receptors. If the red/UV-A increase in *CHS* expression is abolished in a mutant lacking one of these light receptors this would indicate that that specific photoreceptor is involved in mediating the response. It would be expected that the removal of CRY1 and one of the phytochromes would abolish the response.

4.6.1 Red/UV-A Synergism in the Absence of Cryptochrome 1

The *hy4* (cryptochrome 1) mutant was tested under inductive conditions, red followed by UV-A light. In Section 3.8.1 we saw that the expression level of *CHS* in *hy4* mutants was much reduced. Whether that low level of expression can be increased by a red light pretreatment will indicate

whether CRY1 is required for a red light increase in *CHS* expression.

Figure 4.6.1 shows a comparison between the wild type and *hy4* plants treated with red light followed by UV-A light. Plants were exposed to either 100 μmol m^{-2} s^{-1} of red light for six hours or 75 μmol m^{-2} s^{-1} of UV-A light for six hours. A separate set of plants were first exposed to three hours of red light followed by six hours of UV-A at the same fluence levels the single light treatment plants were exposed to. It can be seen that the *hy4* plants show a much reduced level of *CHS* expression in UV-A light, as was seen previously. However, careful observation also shows that there is a very small increase in the level of *CHS* expression seen in the *hy4* plants treated first with three hours of red light before a UV-A treatment. The residual response to UV-A that can be seen in the *hy4* plants, is likely to be due to the CRY2, which also absorbs in the blue/UV-A spectrum. That only a very small increase of *CHS* expression is still retained after a red light pretreatment of *hy4* indicates that the increase is mainly mediated by CRY1. CRY2 may also be able to mediate a small increase in *CHS* expression in the absence of CRY1.

4.6.2 Involvement of Phytochromes in the Red/UV-A Increase

It is likely that phytochromes are involved in the red/UV-A synergistic response. In *Arabidopsis* there is a gene family of five phytochromes, named A to E. The different phytochromes are involved in different aspects of red light control. Phytochromes A and B are the most abundant phytochromes, with PHYA being in the highest abundance in dark grown seedlings and PHYB having the highest abundance in light grown plants (see Section 1.2.1). The aforementioned *phyA*, *phyB* single mutants and the *phyAphyB* double null mutant can be used to investigate the role of phytochromes in the red light pretreatment increase of *CHS* expression.

4.6.3 Red Pretreatment in the Absence of Phytochrome A or B

Phytochrome A is the most intensively studied of the phytochromes and has been shown to be involved in interactions with cryptochrome. Wild

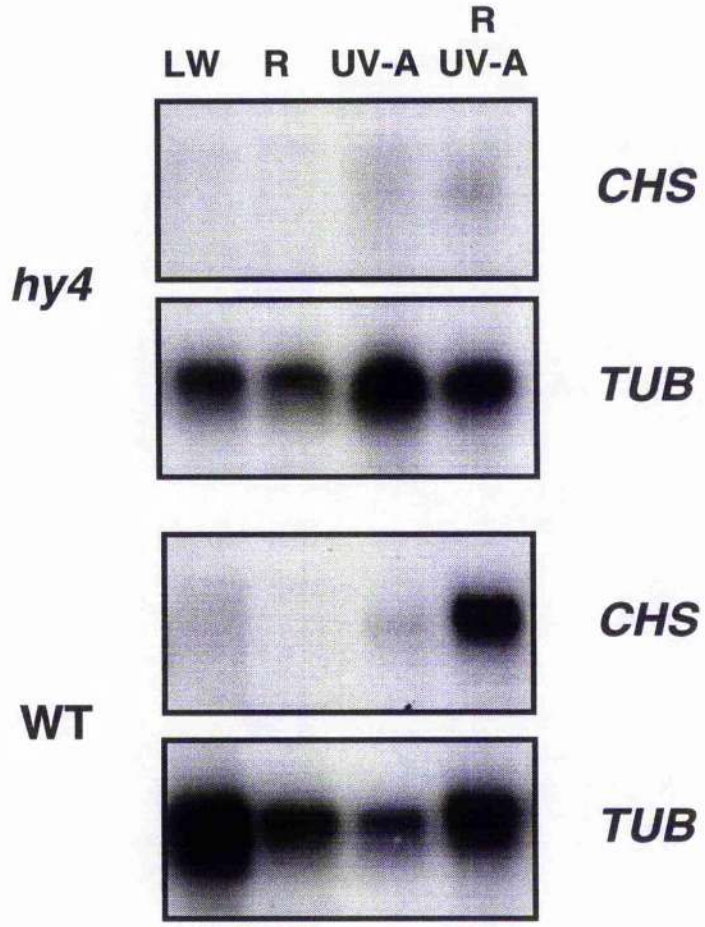


Figure 4.6.1 *CHS* expression increase in response to a red pretreatment before UV-A light exposure requires CRY1

Three week old low white light grown wild type and *hy4* plants were transferred into either six hours red light at 100 µmol $m^{-2} s^{-1}$ (R) or 75 µmol $m^{-2} s^{-1}$ UV-A light (UV-A), or were transferred from red light treatment after three hours into six hours of blue/UV-A light (R UV-A). Untreated plants remained in low white light (LW). Total RNA was isolated from the leaves.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe.

type dark grown seedlings show an enhancement of blue light mediated phototropism when exposed to a brief red light treatment prior to the blue light stimulus; this was not seen in the *phyA* seedlings (Parks *et al.*, 1996).

Looking at *CHS* expression in mature plants treated with red and UV-A light we see a different response. *phyA*, *phyB* and wild type plants were exposed to a three hour red light treatment at 50 μmol m^{-2} s^{-1} prior to a six hour UV-A light treatment at 80 μmol m^{-2} s^{-1} (Figure 4.6.2). This red light pretreatment results in an increase in *CHS* expression in the *phyA* and *phyB* mutants above that seen in UV-A light alone. This implies that phytochromes A and B are either not involved in this synergistic response, or that phytochrome A or B has functional redundancy in this response; that is, if one phytochrome is absent other can take over or have overlapping mediation of this response. It may be that PHYA and PHYB both regulate the red light increase and in the absence of one, the other can function sufficiently to produce a response. This possibility can be tested with the *phyA phyB* double mutant.

If the double mutant lacking in phytochrome A and phytochrome B shows abolition of the red/UV-A synergism this would imply that it is PHYA or PHYB mediated, however if the synergism is retained, this may be due to the synergism being mediated by one of the other phytochromes or that there is functional redundancy and that the synergism is also mediated by one of the phytochromes C, D or E in the absence of phytochromes A and B.

4.6.4 Red Pretreatment in the Absence of PHYA and PHYB

Figure 4.6.3 is a northern analysis of *phyA* and *phyB* single mutants, *phyA phyB* double mutant and wild type plants in red, UV-A and red before UV-A light. Plants were transferred from low white light into six hours of red light at 60 μmol m^{-2} s^{-1}, six hours of UV-A light at 80 μmol m^{-2} s^{-1}, or three hours of red light at 60 μmol m^{-2} s^{-1} prior to the six hour UV-A treatment. It can be seen that in the *phyA* and *phyB* single mutants the red light enhancement of

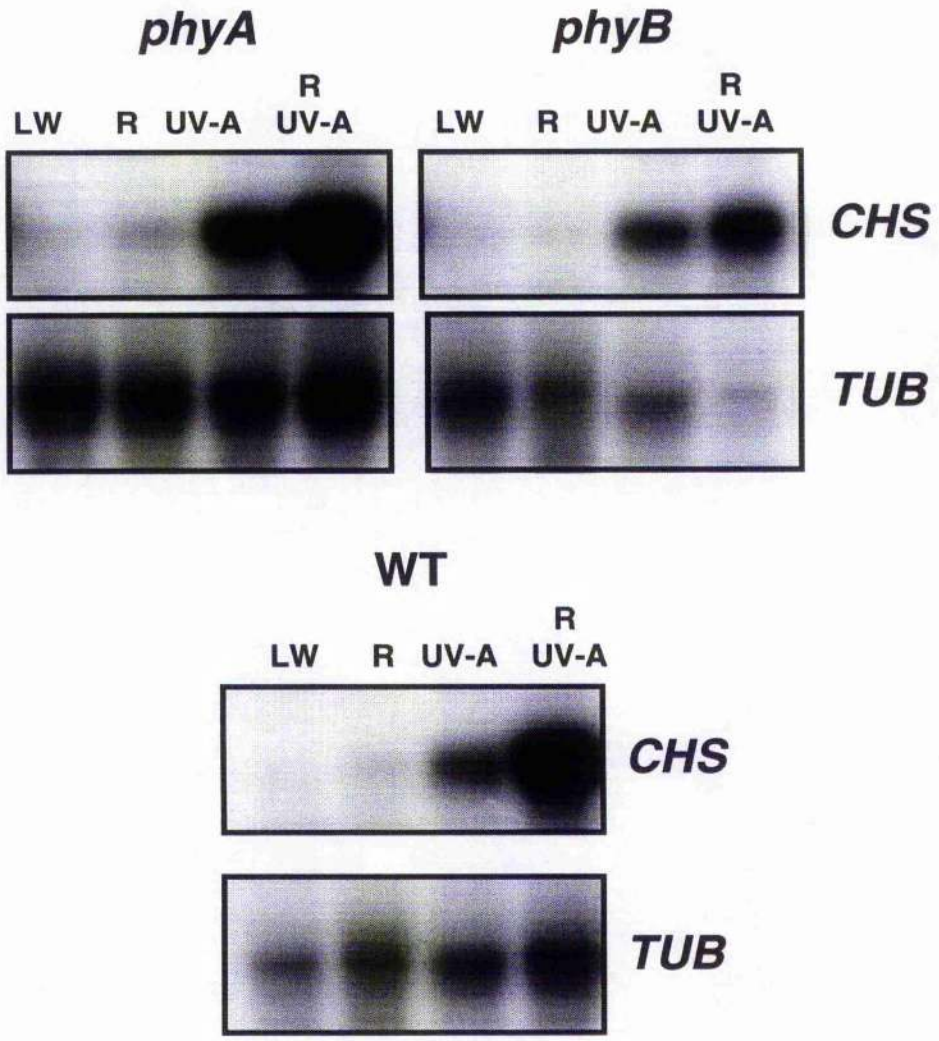


Figure 4.6.2 Red/UV-A synergism is retained in *phyA* and *phyB* mutants

Three week old plants were transferred from low white light (20 µmol m^{-2} s^{-1}) into either red light at 50 µmol m^{-2} s^{-1} (R) or UV-A light at 80 µmol m^{-2} s^{-1} (UV-A) for six hours before harvesting or were transferred from red light after 3 hours into UV-A light and harvested after 6 hours (R UV-A). Total RNA was isolated from leaf tissue.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe.

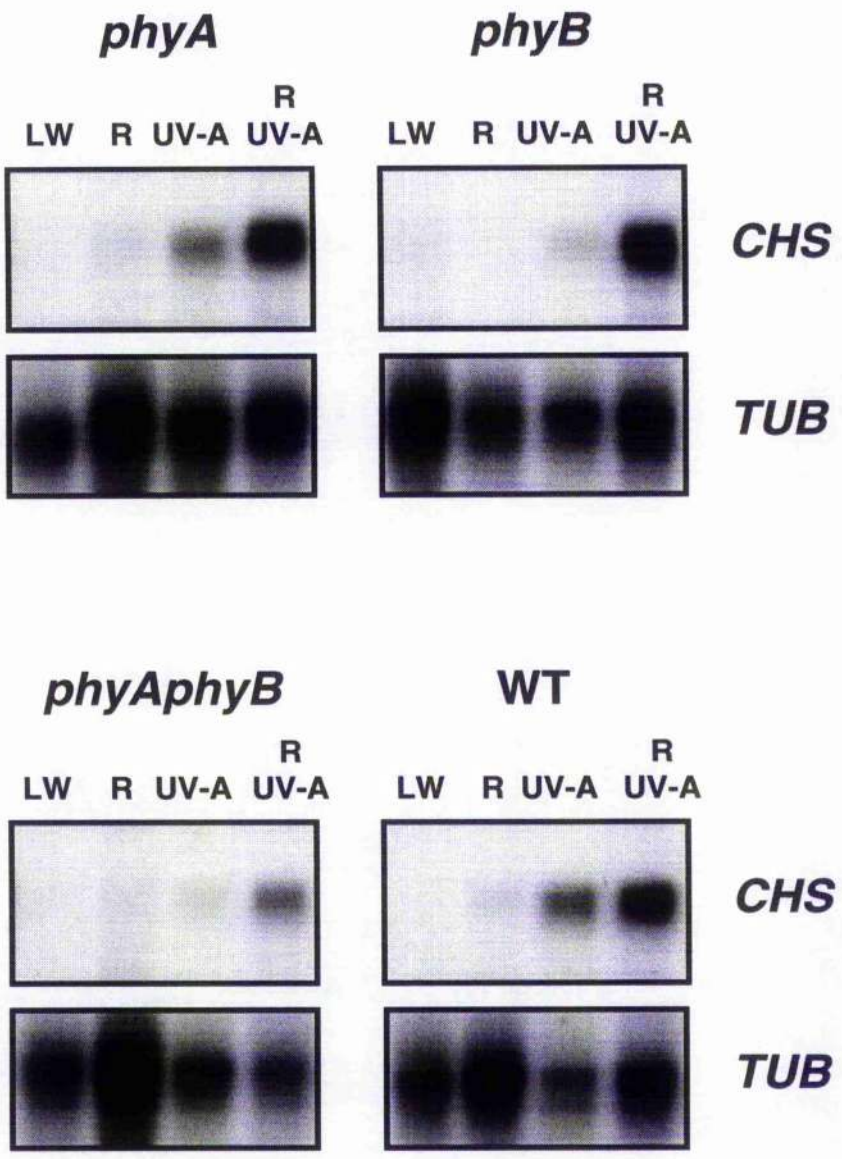


Figure 4.6.3 Red/UV-A synergism is retained in *phyA*, *phyB* and *phyAphyB* mutants

Three week old plants were transferred from low white light (20 µmol m^{-2} s^{-1}) into red light at 60 µmol m^{-2} s^{-1} (R) or UV-A light at 80 µmol m^{-2} s^{-1} (UV-A) for six hours before harvesting or were transferred from red light after 3 hours into UV-A light and harvested after 6 hours (R UV-A). Control plants were untreated (LW). Total RNA was isolated from leaf tissue.

Equal amounts of RNA were separated on a 1.3% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with an *Arabidopsis* chalcone synthase (*CHS*) probe, washed and subjected to autoradiography. Subsequently, the membrane was stripped of radioactivity and reprobed with an α–tubulin (*TUB*) probe.

the UV- A treatment is retained, as before. In the *phyA phyB* double mutant we also see an enhancement of the UV-A light signal by red light. Thus the red light enhancement of UV-A light mediated *CHS* expression does not require phytochrome A or phytochrome B. Nevertheless, the level of *CHS* expression is reduced in the double mutant in red followed by UV-A light, indicating that phytochrome A or B is involved in the response in wild type plants.

4.7 Discussion

The data presented in this chapter show that phytochromes are involved in the regulation of *CHS* gene expression downstream from UV-A and UV-B light signals. Phytochrome B appears to be involved in mediating the signal from UV-A light which results in increased *CHS* expression, as the *phyB* mutant displayed a decrease in *CHS* expression in UV-A light. It did not however account for the total level of expression, as *CHS* expression in *phyB* mutants exposed to UV-A light was greater than that seen in low white light. Similarly, in the *hy4* mutant a decrease in *CHS* expression is seen in UV-A light (Figure 3.8.2). Thus it is likely that *CHS* expression in UV-A light is regulated by an interaction between cryptochrome and phytochrome B. This interaction is potentially that of the phosphorylation of cryptochrome reported by Ahmad *et al.* (1998b) seen in blue light. As cryptochrome is the main photoreceptor for both blue and UV-A light the effect of a potentiating interaction between phytochrome and blue light would be expected to be seen between phytochrome and UV-A light.

In UV-B light induced *CHS* expression, again there is a role played by phytochrome B. In this case, in the absence of phytochrome B we see an increase in the level of *CHS* expression obtained in three week old light grown *Arabidopsis* plants. The removal of phytochrome B produces an increase in *CHS* expression, which is opposite from that seen in UV-A light. If phytochrome B interacts with a UV-B photoreceptor, it must be having the opposite effect to that on cryptochrome. It is also possible that the removal of phytochrome B

results in an increase in UV-B photoreceptors, though there is no obvious reason why this should be. One possible explanation from a survival standpoint is that it may be more effective for plants to produce more sinapic acid ester compounds than flavonoids in the presence of high fluence rates of UV-B light. Mutant plants lacking sinapic acid esters were found to be more sensitive to UV-B damage than mutants lacking either *CHS* or *CFI* gene expression (Landry *et al.*, 1995). The levels of flavonoid types in a given plant alter as a result of UV-B irradiation (Bornman *et al.*, 1997). In wild type plants, phytochrome B may play some role in detecting UV-B light and regulating the expression of *CHS* transcripts. This transcript regulation would act to ensure that the level of CHS protein does not become too high and that both flavonoids and sinapic acid esters are produced. If this scenario is correct, it implies that phytochrome B acts as a negative regulator of *CHS* expression in UV-B light, moderating the level of response to a given light treatment.

That we see an effect of phytochrome B and not any real difference in the *phyA* mutant in both UV-A and UV-B light may be due to the growth conditions of these plants. These mature plants are grown in constant low white light. The light labile phytochrome A is only present in low quantities, PHYB is not light labile and therefore is likely to be present in higher quantities than PHYA in these three week old plants. A greater effect due to phytochrome A may be seen in dark adapted plants. This was not investigated here.

In these plants the involvement of phytochrome B in regulating *CHS* expression is further reinforced by the red light pretreatment increase of *CHS* expression in UV-A light. Red light treatment prior to UV-A light exposure results in an increase in *CHS* expression. However, this increase remains in both the *phyA* and *phyB* single mutants and in the *phyA phyB* double mutant plants. The actual levels of *CHS* expression in UV-A light are decreased in *phyB* and *phyA phyB* mutants, but as was seen in Figure 4.6.3 an increase in the level of expression was still seen after red light pretreatment. This indicates that while phytochromes are involved in regulating the expression level of *CHS* in

UV-A light, the red light pretreatment increase can be mediated by something other than PHYA or PHYB. The possible explanations for the increase in *CHS* expression in the red light treated plants are that phytochromes C, D or E mediate the response or that metabolic intermediates are involved. Red light is sufficient for photosynthesis and therefore the level of sucrose in the red light pretreated plants may be higher than in the plants transferred directly from low white light into UV-A light. The effect of increased sucrose has been noted to alter the level of *CHS* expression seen in wild type *Arabidopsis* plants (Tsukaya *et al.*, 1991; Valentine, 1998, and Section 3.7). This increased sucrose content of the plants may be sufficient to cause an increase in *CHS* expression in the red pretreated plants. Additionally, with the increase in photosynthesis there will be an increased flux through the shikimate pathway. Loake (1991) reported an increase in *CHS* expression seen after elicitor induction in the presence of metabolic intermediates. In plants treated with red light prior to *CHS* induction, the increased levels of metabolic intermediates may promote the increased *CHS* expression seen under the UV-A inductive conditions.

Chapter 5: Screen for Mutants Altered in Positive Regulators of *CHS* Expression

5.1 Introduction

icx1 was isolated in a screen for mutants with altered *CHS* expression in white light. *icx1* is a negative regulator of *CHS* expression and was isolated due to the mutant having a higher accumulation of GUS driven by a *CHS* promoter in response to light (see Jackson *et al.*, 1995, Introduction and Chapter 3). Using this *CHS-GUS* transgene screen it should be possible to isolate both negative and positive regulators of *CHS* expression. However, the mutant screen reported by Jackson *et al.* (1995) produced two mutants with increased levels of *CHS* expression in response to light, but none with decreased levels. Moreover, the use of a GUS assay did not permit a large scale screen for putative mutants. Thus in order to specifically find mutants in positive regulators of blue/UV light induction a different sort of screen should be devised. An efficient screen which is targeted to isolate mutants altered in positive regulators of *CHS* expression should permit the rapid identification of plants with a lower level of *CHS* expression under inductive conditions.

It is possible to design a mutant screen which discriminates against plants that express a high level of *CHS* expression in inductive conditions. In order to do this, a gene product can be utilised to convert a substrate into a toxic product. If the gene is driven by a *CHS* promoter, the amount of gene product is regulated in the same manner as *CHS* itself. Thus, plants expressing high levels of *CHS* accumulate a greater amount of the toxic product, and low expressers accumulate less. The mutants expressing low levels of *CHS* have a greater chance of survival, and can be rescued from the selection media.

5.2 Suicide Screen

In order to isolate mutants with low levels of *CHS* expression in inductive fluences of light, we decided to develop a transgene expression

screen utilising cytosine deaminase (CD). CD is present in prokaryotes and many eukaryotic micro-organisms, it is not normally found in higher plants. CD catalyses the deamination of 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU). 5-FU is the precursor of 5-fluoro-dUMP (5-F-dUMP). 5-F-dUMP is toxic to cells as it irreversibly inhibits thymidylate synthase (Beck *et al.*, 1972). Thymidylate synthase is inhibited by 5-F-dUMP when 5-F-dUMP acts as a substrate for the enzyme. 5-F-dUMP cannot then be removed from thymidylate synthase. Thymidylate synthase is particularly active in cells which are rapidly dividing, such as in seedlings, as the enzyme is involved in DNA synthesis. Inhibition of thymidylate synthase is therefore deleterious to the health and growth of a plant.

CD has been used in a number of mutant screens, including that of Serino and Maliga who introduced CodA (the cytosine deaminase coding sequence) into tobacco plastid genomes as the basis for a screen to identify nuclear genes which control plastid gene expression (1997). Kobayashi *et al.* (1995) introduced CD driven by the 35S Cauliflower mosaic virus promoter into *Arabidopsis*. The sensitivity of the transgenic descendent plants to 5-FC indicated that CD is useful as a means of negative selection.

Cytosine deaminase can be used in a transgene expression screen to identify mutants with low levels of *CHS* expression. A suicide expression screen can be created by introducing CodA driven by the *CHS* promoter. The *CHS-CD* cassette is illustrated in Figure 5.2.1. Once *CHS-CD* is stably introduced into *Arabidopsis* and seeds mutagenised the seed population can be screened on plates containing 5-FC. Mutants expressing high and normal levels of *CHS* will convert large amounts of 5-FC into 5-FU and will stop growing or die. Mutants which have low levels of *CHS* expression and thus low levels of cytosine deaminase will not convert as much 5-FC into 5-FU and while the plants may be sickly, they will survive longer than the plants expressing high or normal amounts of *CHS-CD* under the same inductive conditions. Low expressing mutants can be rescued by transferring seedlings onto fresh plates

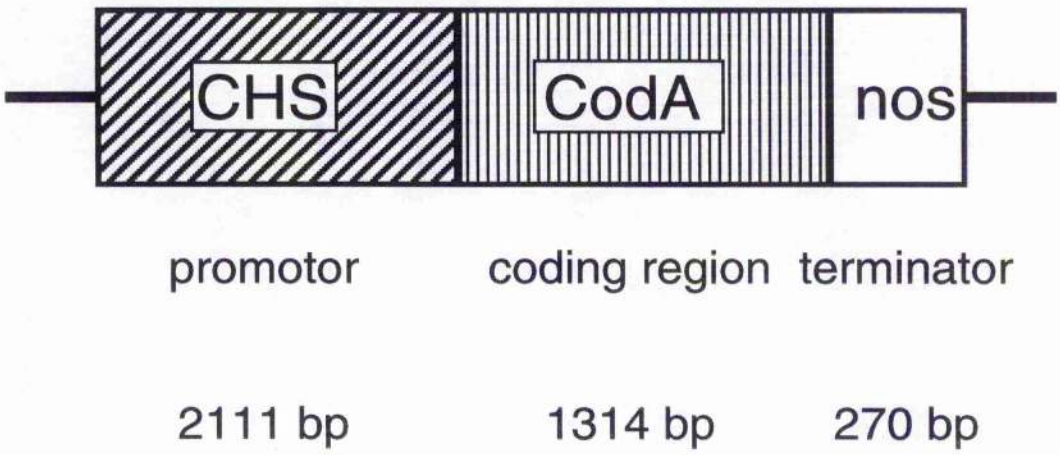


Figure 5.2.1 Diagram of the*CHS-CD* transgene
Model of the transgene to be introduced into wild type plants, to provide the basis of a mutant screen for positive regulatory mutants of *CHS* induction. CHS - chalcone synthase promoter, CodA - the cytosine deaminase coding sequence.The sizes of the sequences are given in base pairs (bp) corresponding to the sequence named above.

which do not contain 5-FC.

The method of construction of the *CHS-CD* transgene and its introduction into *Arabidopsis* plants is described in this Chapter.

5.3 Transgene Construction

A schematic diagram of the construction of the *CHS-CD* transgene in pBluescript is illustrated in Figure 5.3.1. The plasmids from which the required DNA was obtained were as follows:- the *CHS* promoter from the *CHS* promoter-GUS containing plasmid p459 (Ulrike Hartmann, provided by Bernd Weisshaar, MPI, Köln); CodA from pMSCD, a plasmid containing the *CD* coding region (provided by Ian Graham, Glasgow University, Glasgow). pMSCD is based in the pBluescript vector and has a malate synthase promoter, which was to be replaced by the *CHS* promoter. The restriction sites available to cut out the *CHS* promoter and to remove the *MS* promoter from pMSCD were not compatible for direct ligation. Klenow 5′ fill in allowed blunt end ligation of one fragment end and further digestion produced compatible ends of the pBluescript fragment with the *CHS* promoter fragment. The method of cloning is described in detail below.

5.3.1 pCHSCD Cloning

The *CHS* promoter was cut out of the *CHS-GUS* containing plasmid, p459, using the sites *Hin*dIII and *Nae*I in a sequential digest. The digest was run out on a 0.8% TAE gel. Figure 5.3.2 is a picture of the gel, the *CHS* promoter fragment is indicated with an asterix. This *CHS* promoter fragment of 2111 bp was cut out and extracted from the gel as described in Section 2.9.

The cytosine deaminase coding sequence, in the pMSCD plasmid consisted of the malate synthase (*MS*) promoter in front of the cytosine deaminase coding sequence cloned into pBluescript. The *MS* promoter and the *CodA* sequence had been inserted into pBluescript in the multiple cloning site (MCS) at the *Bam*HI site. If the pMSCD plasmid was digested to completion with *Bam*HI the entire *CodA* sequence would be cut out. The MS promoter was

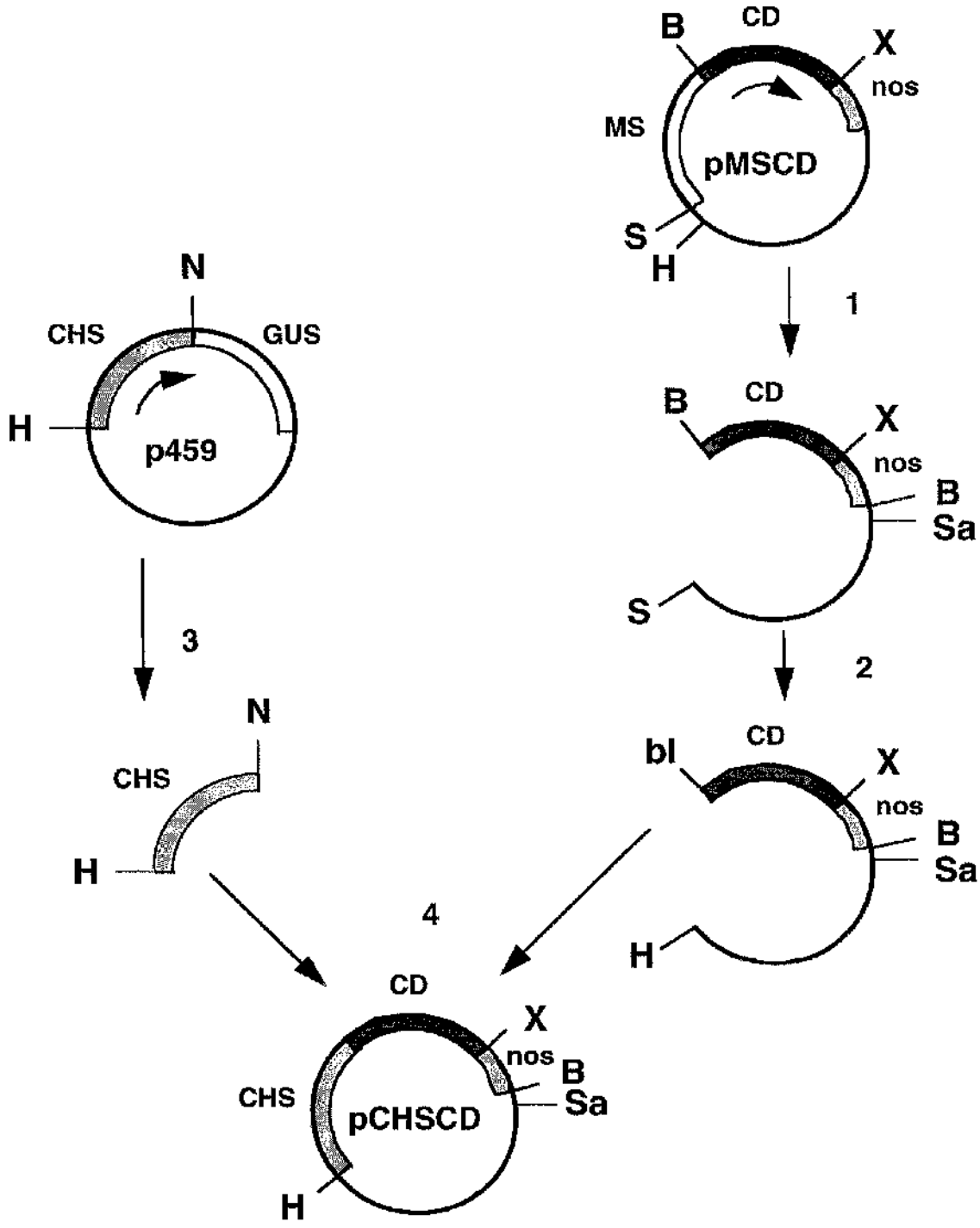


Figure 5.3.1 Construction of the CHS-CD transgene

The steps used to produce the CHS-CD transgene in the pBluescript vector are as follows: 1) the Malate synthase promoter was removed from the pMSCD plasmid by a 100% SmaI digest followed by a partial *Bam*HI digest. The fragment was gel purified prior to 2) Klenow treatment of the sticky ends and further digest with *Hin*dIII 3) the p459 plasmid was digested with *Nae*I and *Hin*dIII and the CHS promoter isolated by gel purification. Finally 4) ligation of the two fragments obtained from steps 2 and 3.

B= *Bam*HI, bl= blunt end, H= *Hin*dIII, N= *Nae*I (blunt end), S= *Sma*I, Sa= *Sac*I, X= *Xba*I.

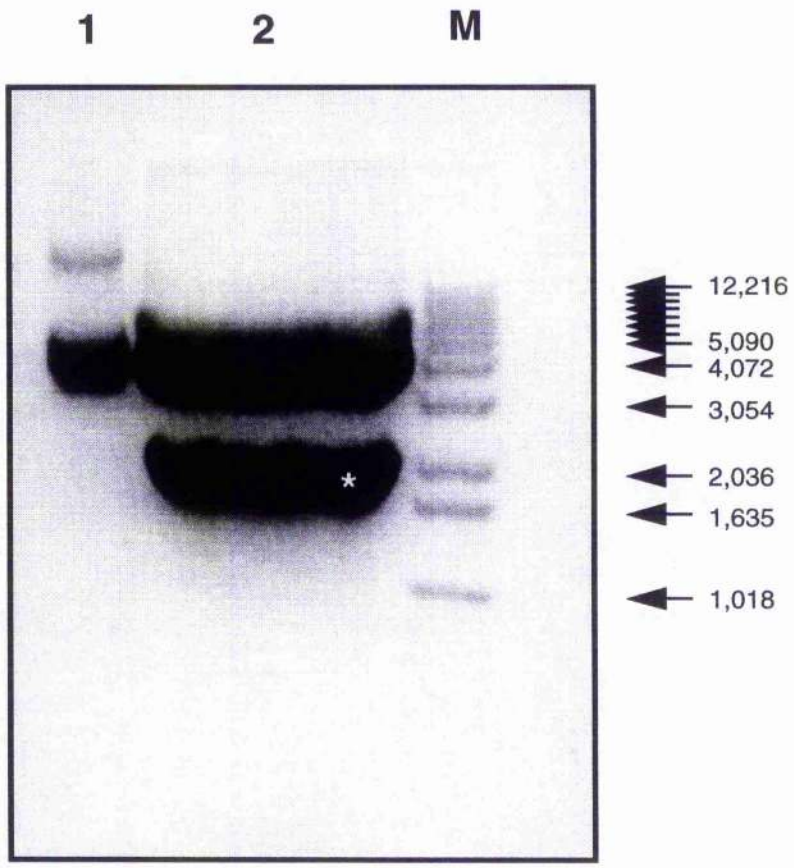


Figure 5.3.2 p459 digest to isolate the *CHS* promoter

The 2111 bp fragment corresponding to the *CHS* promoter was cut out of the p459 plasmid with *Hin*dIII/*Nae*I. This fragment is indicated by the asterix (*).

lane 1; undigested p459: lane 2; p459 digested with *Hin*dIII/*Nae*I: M; marker, 1kb ladder, some of the the sizes are indicated at the right of the gel.

The gel purified *CHS* promoter fragment from this gel is shown in lane 3 of Figure 5.3.4.

The DNA was electrophoresed on a 0.8% TAE gel containing ethidum bromide and visualised on a UV transilluminator.

removed from pMSCD by a 100% *Sma*I digest and a partial *Bam*HI digest. A partial *Bam*HI digest was made after an initial linearising *Sma*I digest which cuts once, in front of the *MS* promoter in the MCS.

2 µl of the *Sma*I only digest was run out on the same gel as the partial digest to check whether the plasmid had fully linearised and to compare with the *Bam*HI digest. The partial digests were made in two separate "master mixes" (described in Appendix I) and aliquots removed after 0, 5, 10, 20, 30, 40, 50, and 60 minutes and the reaction stopped by addition of loading buffer. This partial digest produced a number of fragments which were run out on an 0.8% TAE gel to separate the differently sized products, as can be seen in Figure 5.3.3. The expected products were:-

5359 bp linear pMSCD, undigested by *Bam*HI

4275 bp *Bam*HI-*Sma*I fragment, *CodA* and vector

2961 bp *Bam*HI-*Sma*I fragment, vector only

2398 bp *Sma*I-*Bam*HI fragment, *MS-CD* cassette

1314 bp *Bam*HI-*Bam*HI fragment, *CodA*

1084 bp *Sma*I-*Bam*HI fragment, *MS* promoter

The fragment corresponding to the correct size for the pMSCD fragment minus the *MS* promoter (4275 bp) was cut out of the gel and extracted from the gel slice. The fragment cut out is indicated by an asterix in Figure 5.3.3.

The *CHS* promoter could not be cloned directly in front of the *CD* gene as the *CHS* promoter fragment included the start site of the *CHS* gene from p459. This was due to an absence of restriction sites close to the *CHS* ATG site. 70 bp of the *CHS* coding region is included with the *CHS* promoter fragment. The additional bases included in the *CHS* promoter are noted in Appendix II. In order to keep the start site of the *CHS* gene in frame with that of the cytosine deaminase the following procedure was used. The 4275 bp fragment from pMSCD (plasmid plus the *CodA*) was treated with Klenow to produce a 5′ fill in (Section 2.10.3) resulting in blunt ends instead of the sticky ended fragments produced by the *Sma*I/*Bam*HI digest. This blunt ended product of 4282 bp was

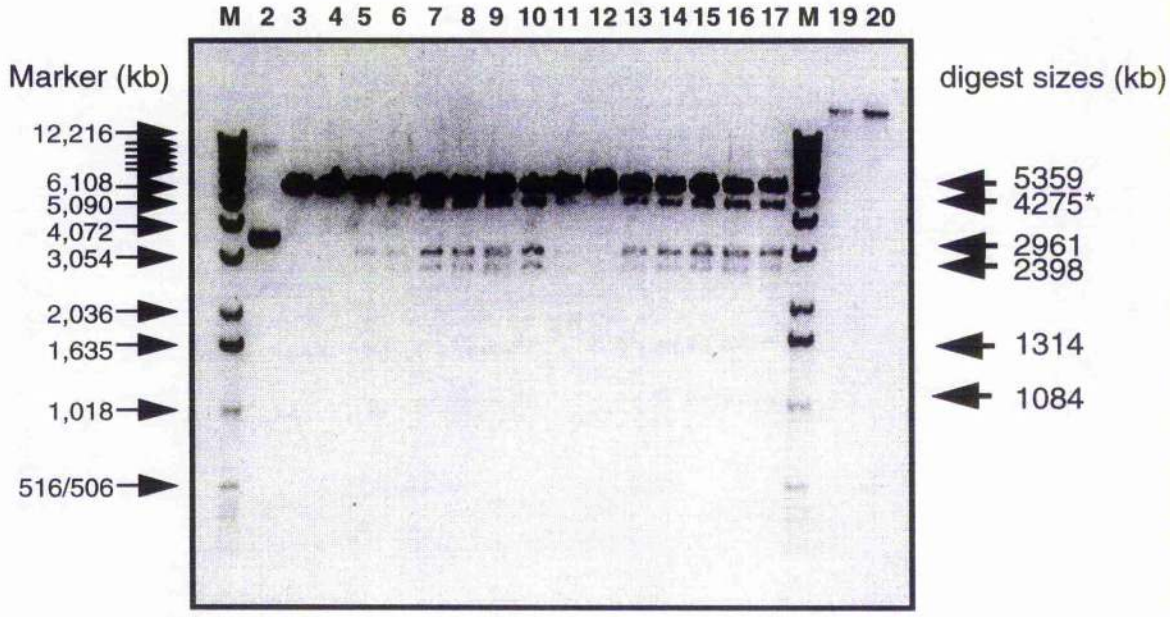


Figure 5.3.3 partial digest of pMSCD

As described in step 1 of Figure 5.3.1

The fragment cut out of the gel was the 4275 bp fragment indicated by a * and is made up of the Bluescript vector and the cytosine deaminase coding region, the malate synthase promoter is removed.

The lanes contain DNA as follows:- lanes M, 1 kb marker; lane 2, uncut pMSCD; lane 3, *Sma*I digest only; lanes 4 & 11, 5 min digest; lanes 5 & 12, 10 min digest; lanes 6 & 13, 20 min digest; lanes 7 & 14, 30 min digest; lanes 8 & 15, 40 min digest; lanes 9 & 16, 50 min digest; lanes 10 & 17, 60 min digest; 19, 25ng and 20, 50 ng lambda DNA.

The DNA was electrophoresed on a 0.8% TAE gel containing ethidum bromide and visualised on a UV transilluminator.

then digested with *Hin*dIII to produce a fragment with one sticky end while the other end remained blunt. The sticky end could then be ligated to the *Hin*dIII sticky end of the *CHS* promoter fragment. The Klenow treated blunt end could be ligated to the blunt end from the *Nae*I digest of the *CHS* promoter giving a plasmid of ~6.3 kb with the *CHS* promoter in front of the *CodA* sequence, with both in the correct orientation and in frame.

The *CHS* promoter, *CD* plus plasmid ligation mix was used to transform competent cells which were then streaked onto LB and kanamycin selection plates. The colonies obtained were used to inoculate a 3 ml overnight culture and the plasmids from these colonies isolated and digested with *Xba*I and *Hin*dIII restriction enzymes. The colonies with *CHS-CD* in the correct orientation and a successful Klenow 5' fill in will cut once with *Hin*dIII and once with *Bam*HI. Figure 5.3.4 shows the results of the *Bam*HI/*Hin*dIII digest run out on a 0.8% TAE gel. In all plasmids isolated two fragments can be seen. The expected fragment sizes are 3699 bp for the *CHS-CD* cassette (including the nos terminus) and 2.9 kb for the pBluescript plasmid. The sizes of the fragments obtained from the digests correspond to the expected sizes of the pCHSCD plasmid. This indicates that the ligation was successful. One of these colonies was chosen to sequence to ensure that the *CHS* promoter with the initial *CHS* coding sequence and the initial coding sequence of cytosine deaminase were in frame.

5.3.2 Sequencing of pCHSCD

The sequence was checked by ^{35}S labelled dideoxy chain termination sequencing and found to be in frame. The oligonucleotide obtained from Cruachem (its sequence can be found in Appendix II) read from within the *CHS* promoter, allowing sequencing through the *CHS* coding region and across the border to the *CodA*. Figure 5.3.5 indicates the area sequenced. The sequence obtained was as expected and is shown in Appendix II.

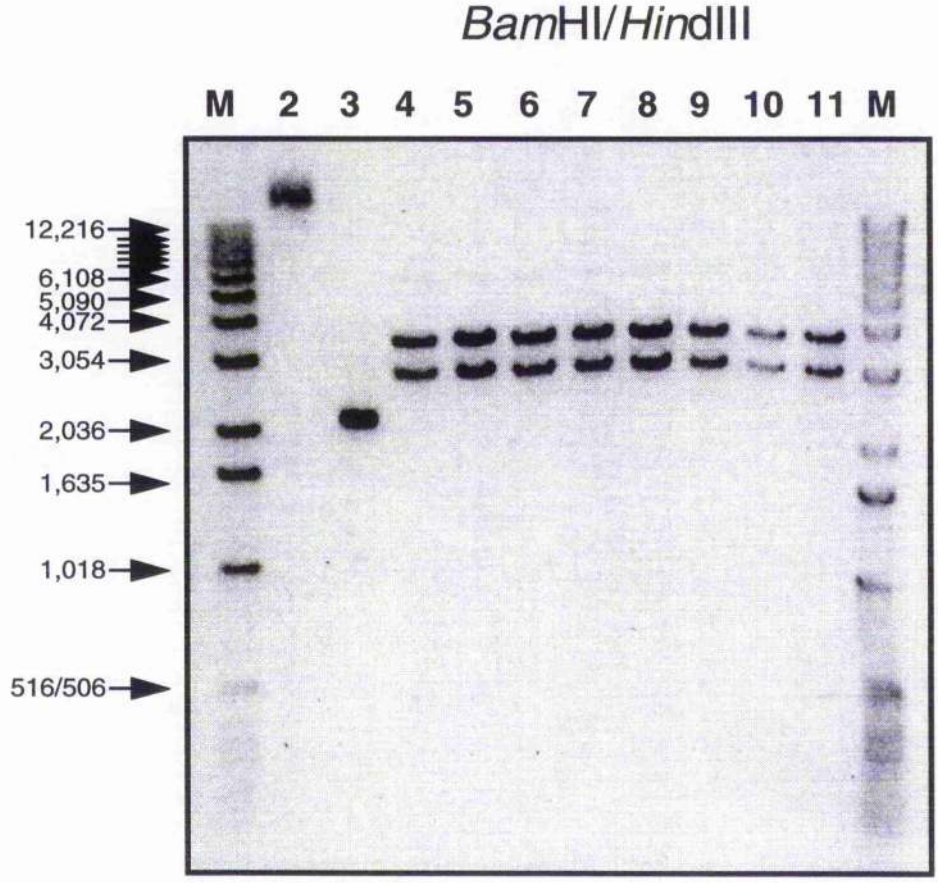
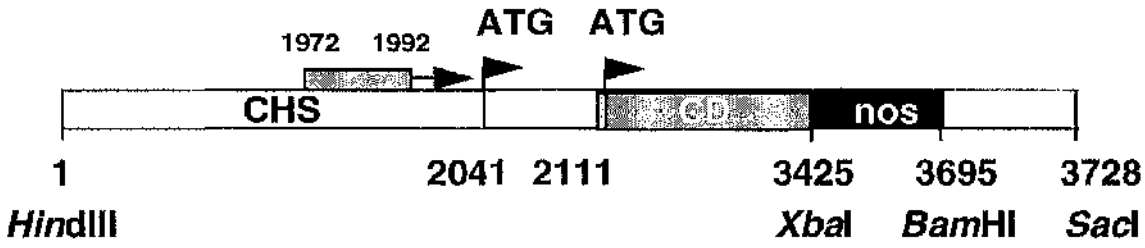


Figure 5.3.4 pCHSCD digest with *Bam*HI/*Hin*dIII

To check whether the colonies picked from the selection plates were the expected CHSCD construct, 2 ng of the plasmid from each colony were digested with *Bam*HI and *Hin*dIII. This should produce two fragments, one of 3695 bp (the CHSCD cassette) and one of 2.9 kb (the pBluescript vector). All the colonies contained the vector and cassette.

The loading was as follows:-lanes M, 1 kb ladder; lane 2, uncut 4.2; lane 3, *CHS* promoter only. The following lanes contained the plasmids indicated digested with *Bam*HI and *Hin*dIII:-lane 4, p4.2; lane 5, p4.5; lane 6, p4.6; lane 7, p4.10; lane 8, p4.11; lane 9, p4.12; lane 10, p4.14; lane 11, p4.16.

The DNA was electrophoresed on a 0.8% TAE gel containing ethidum bromide and visualised on a UV transilluminator.



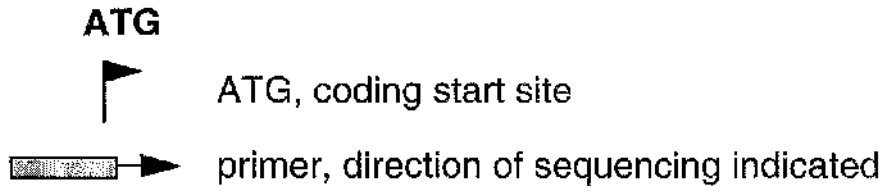


Figure 5.3.5 The region of pCHSCD sequenced
The 20mer primer used annealed to a region in the CHS promoter between 49 and 29 bp infront of the CHS start site, the area to be sequenced was that of the ligation between CHS and CD. Numbering the CHS-CD cassette bases from the *Hin*dIII digest at the end of the promoter (1) to the SacI digest at 3728. The diagram is not to scale.

5.4 Introduction of *CHS-CD* into *Agrobacterium*

5.4.1 pBI101 Ligation

The *CHS-CD* cassette was cut out of the pBluescript plasmid and ligated into pBI101 in order provide the left and right borders needed to insert the *CHS-CD* into plants. pBI101 was digested with *Hin*dIII and *Sac*I in a sequential digest, removing the GUS gene and the nos terminator and the large fragment was gel purified (Figure 5.4.1). pCHSCD with it's nos terminator was also digested out of the pBluescript plasmid with *Hin*dIII and *Sac*I, and the 3728 bp fragment was gel purified (data not shown). The pBI101 fragment and the *CHS-CD* fragment were ligated and transformed into *E. coli* (as described in Sections 2.10.2 and 2.6.2) and spread on a YEP plate containing 50 mg/ml kanamycin. The plates were left to incubate overnight and colonies were picked the next day to inoculate overnight cultures. These overnight cultures were then used to make small scale plasmid preps, to test the plasmid by restriction mapping.

2 µl of the putative pBICHSCD plasmids were digested with *Hin*dIII/*Sac*I and then run out on a 0.8% TBE gel. In the *Hin*dIII/*Sac*I digests two fragments were present, corresponding to the *CHS-CD* cassette (3728 bp) and the pBI101 plasmid (10.07 kb).

5.4.2 *Agrobacterium* Transformation

Once it had been checked by restriction fragment mapping that the *CHS-CD* cassette was in the correct orientation in the pBI101 plasmid (pBICHSCD) the pBICHSCD was transformed into transformation competent *Agrobacterium*, strain GV3101, which is rifampicin and gentamycin resistant. The pBICHSCD plasmid is kanamycin resistant. Transformation of pBICHSCD into *Agrobacterium* strain GV3101 was completed as described in Section 2.6.4.

After transformation, the *Agrobacterium* were plated out on kanamycin and gentamycin selection plates. Six colonies were picked to inoculate 3 ml overnight cultures. Plasmid preps were made of the six cultures, 2 µl of each were digested with *Hin*dIII or with *Hin*dIII/*Sac*I. The digests were then run out

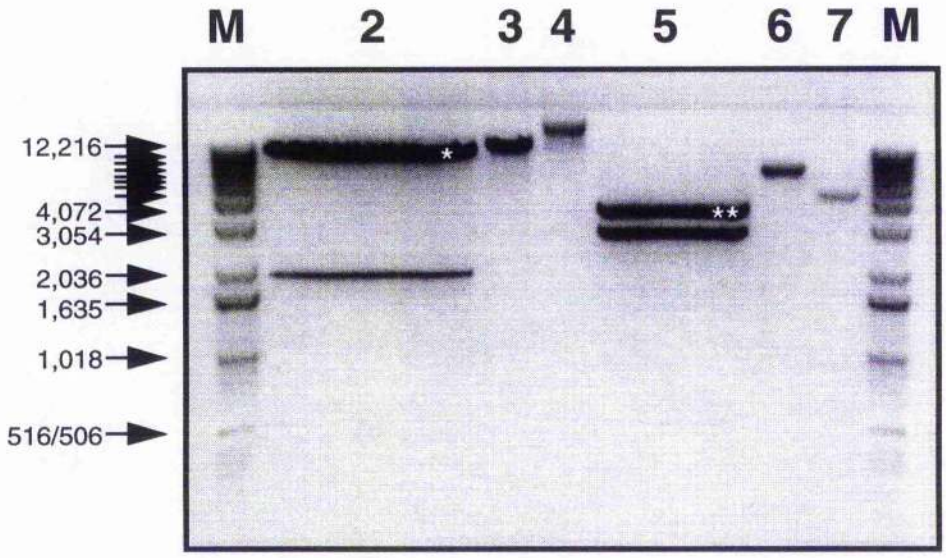


Figure 5.4.1 pBI101 and pCHSCD digests for ligation

The *CHSCD* cassette was cut out of the pCHSCD with a *Hin*dIII/*Sac*I double digest (lane 5), the pBI101Agrobacterium binary vector was also digested with *Hin*dIII/*Sac*I double digest (lane 2), to remove the GUS cassette present. The large 10.07kb fragment of the pBI101(*) and the 3695 bp fragment of the pCHSCD (**) digest were cut out of the gel and purified as described in materials and methods.

The lanes were loaded as follows:-M, 1 kb ladder; lane 2, pBI101 digested with *Sac*I and *Hin*dIII; lane 3, pBI101 *Hin*dIII digest; lane 4, uncut pBI101; lane 5, pCHSCD digest with *Hin*dIII and *Sac*I; lane 6, pCHSCD *Hin*dIII digest; lane 7, uncut pCHSCD.

The DNA was electrophoresed on a 0.8% TAE gel containing ethidum bromide and visualised on a UV transilluminator.

on a gel shown in Figure 5.4.2. It was seen in the single *Hin*dIII digest that a single product was produced, the linearised pBICHSCD. The *Hin*dIII/*Sac*I digest produces two band, one of the *CHS-CD* cassette and one of the pBI101 vector. All six cultures contained the 3728 bp *CHS-CD* fragment. One of these colonies was used to inoculate several 5 ml overnight cultures. The overnight cultures were then used to inoculate two flasks containing 500 ml of LB containing 50 µg/ml kanamycin and 25 µg/ml gentamycin. These cultures were used to vacuum infiltrate *Arabidopsis* plants, which were at the flower bolt stage. Vacuum infiltration of plants was executed as described in Section 2.15.

5.5 Selection of Transformed Seed

The plants which had been vacuum infiltrated were returned to a growth room and placed under low white light, to set seed. After several weeks the plants were left to dry, and the seeds collected. The seeds were surface sterilised (as described in Section 2.3.3) and sown on selection plates (as described in Section 2.15) containing 50 µg/ml kanamycin. Controls of a wild type untransformed line and the kanamycin resistant NM4 line were also sown.

Seedlings displaying elongated hypocotyls and green cotyledons were transplanted from the selection media onto soil and allowed to set seed. Of the fourteen seedlings selected from the selection screen, only four survived to produce seeds.

To ensure that the seedlings produced from this screen contained an active copy of the *CHS-CD* construct, a proportion (approximately 25%) of the seed was tested by growing on plates containing 5-FC.

The seeds were sown on agar containing 1 µg/ml 5-FC, left to germinate in the dark then transferred into high white light. The death rate of the seedlings was monitored against that of non *CHS-CD* containing wild type plants. The survival rate of the plants on 5-FC was equivalent to that seen in the wild type plants, indicating that either cytosine deaminase was not being expressed or that the plants selected were false positives. Sowing of a further

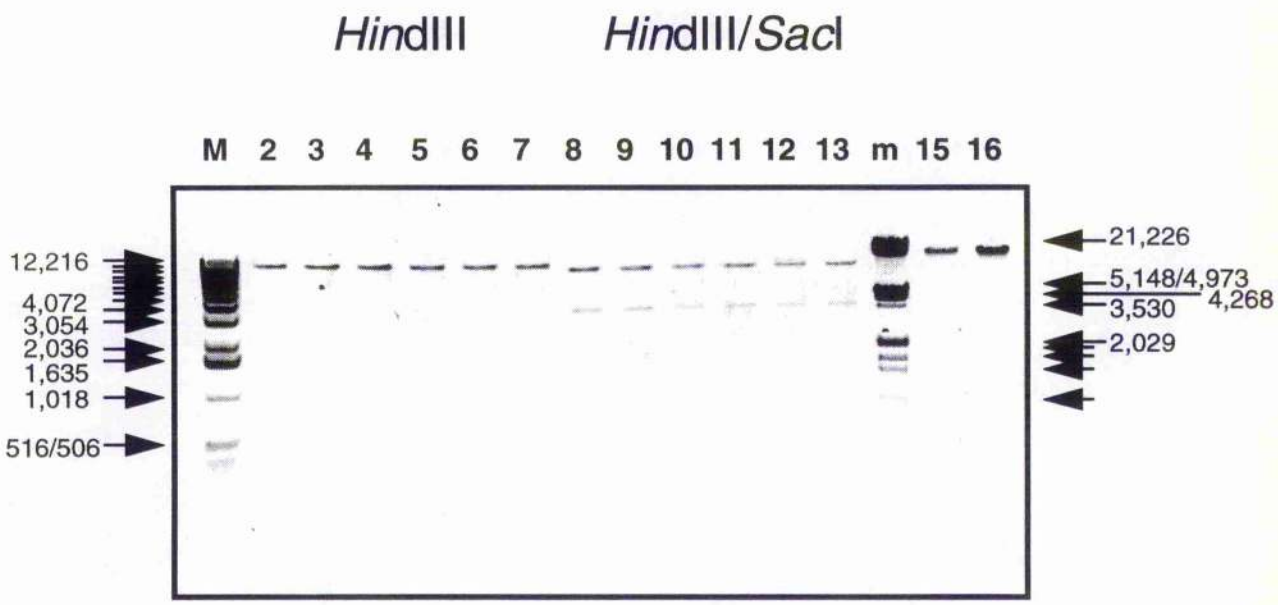


Figure 5.4.2 Digest of p5.4 by *Hin*dIII and *Hin*dIII/*Sac*I

After transformation of *Agrobacterium* with the binary vector containing CHSCD, the colonies obtained were checked for the pBICHSCD plasmid.The six colonies were digested with *Hin*dIII (lanes 2-7) or both *Hin*dIII and *Sac*I (lanes 8-13, colonies loaded in the same order).

The linearized product seen in lanes 2-7 is a result of the *Hin*dIII digest of pBICHSCD, further digestion with *Sac*I produces two fragments, the CHSCD cassette (3728 bp) and the pBI101 (10.07 kb) *Agrobacterium* transfer vector.

lane M; 1 kb ladder lane m; lambda digested with *Eco*I, *Hin*dII
lanes 15 and 16; 25 and 50 ng of lambda DNA.

DNA was electrophoresed on a 0.8% TBE gel containing Ethidium Bromide for visualisation on a UV transilluminator.

25% of the seed on kanamycin plates resulted in 100% of the plants bleaching, indicating that the plants chosen in the first round of kanamycin selection were false positives.

As a result of this setback, further plants were vacuum infiltrated with *Agrobacterium* carrying the *CHS-CD* construct to repeat the selection process. After seed set and harvest approximately 2000 plants were sown on kanamycin selection plates. Five plants were obtained which remained green and produced first leaves while the seedlings around them bleached and died. The plants were left on kanamycin for two weeks to reduce the possibility that they were false positives. During this time the plants grew well and were comparable to the control (kanamycin resistant) plants. Immediately prior to transfer onto soil, the plates were photographed, the transgenic plants can be seen in Figure 5.5.1. These plants will provide the basis of the CD negative selection screen.

5.6 Discussion

The ground work has been done for the continuing work to produce a screen for the selection of positive regulators of *CHS* expression. It is hoped that the putative transgenic plants produced in this screen are genuine CHSCD expressers and not false positives as in the initial screen.

When the transgenic plants are mutated, the screen for low *CHS* expressing mutants will no doubt produce a number of mutants which have already been isolated. Novel mutants should also be forthcoming. The screen has the potential to provide a means to help dissect the pathway from light signals to *CHS* expression. Isolation of mutants in defined light conditions has the possibility to isolate proteins specifically altered downstream from either UV-B or from UV-A/blue light signals. Production of mutants in these pathways will help to elucidate the light signal transduction pathways present in plants.

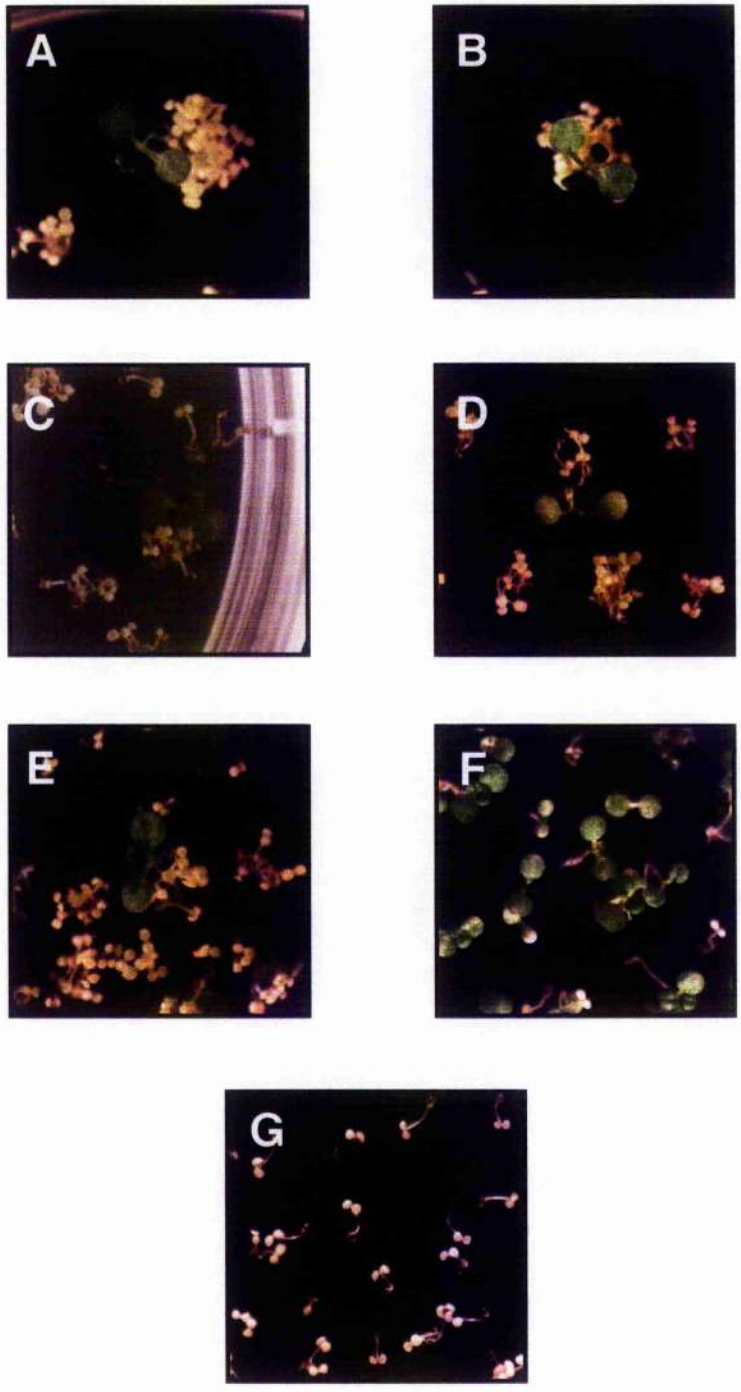


Figure 5.5.1 Seedlings putatively transformed with CHSCD
Seeds were sown on 1/2 MS plus 50 μg/ml kanamycin.
A-E, putative transgenic plants transformed with CHSCD; F, kanamycin resistant NM4 plants; G, kanamycin sensitive wild type plants.

Chapter 6: Final Discussion

6.1 Introduction

From investigation of the model system of *CHS* expression in response to a variety of light and environmental stimuli, the picture that emerges is one of complex interactions between the phototransduction pathways which result in the observed responses. Given the variety of combinations of light fluence and quality a plant would naturally receive, it is perhaps unsurprising that there is interaction between light signalling pathways, mediating the final responses. This interaction between the various downstream components allows the plant to respond with greater plasticity than if a single signal produced a single response of a set magnitude.

A number of aspects of light regulation of *CHS* expression have been investigated in the previous chapters, in the characterisation of *icx1* (Chapter 3) and in the involvement of phytochrome in UV light regulated *CHS* expression in mature plants (Chapter 4). Further investigation of light interactions and signalling intermediates will be possible with a screen for mutants with reduced levels of *CHS* expression, which has been initiated as described in Chapter 5.

6.2 Characterisation of the *Arabidopsis icx1* Mutant

In Chapter 3, and from previous studies, it can be seen that *CHS* expression is affected by a number of different stimuli and that light regulation alters with the developmental stage of the plant. ICX1 acts as a negative regulator downstream from a number of different stimuli.

The *icx1* mutant was shown to have a light mediated increase in *CHS* expression above that of wild type plants (Jackson *et al.*, 1995). It can be seen that ICX1 is involved in regulating *CHS* expression downstream from a variety of signals, but not in all cases tested (for example UV-B plus blue or UV-B plus UV-A light synergism). ICX1 is not just acting as a negative regulator

downstream of a particular photoreceptor signal pathway as the *icx1* mutant displays increased *CHS* expression in far-red, UV-A/blue, UV-A and UV-B light. The presence of ICX1 in the wild type constrains *CHS* expression in response to signals transduced from phytochrome, cryptochrome and UV-B photoreceptor(s). ICX1 appears to act to regulate the level of transcription of *CHS* (and other flavonoid biosynthetic enzymes) in response to these signals downstream from photoreceptors. ICX1 also plays a role as a negative regulator of *CHS* expression in response to cold treatment of plants. Therefore ICX1 does not only act downstream of light signals, though there is a light requirement for the cold increase of *CHS* expression in *Arabidopsis*. The light mediated expression of *CAB* and *rbcS* does not show altered expression in the *icx1* mutant from that of the wild type.

The pleiotropic phenotype of the *icx1* mutant is either a result of an increase in flavonoids accumulating in the plant or due to some other, unidentified, effect of the mutation in ICX1. Spatial visualisation of the location of the *CHS* expression was made via *CHS-GUS* expression and subsequent histochemical staining. We see that in *icx1* the *CHS* expression is not altered in location from that of the wild type (Figure 3.6.1). Ectopic expression of *CHS* and the resultant ectopic flavonoid accumulation is not the cause of the altered visible phenotype.

It appears that while ICX1 acts as a negative regulator in many of the responses investigated here, the conspicuous exception to this is in UV-B plus blue and UV-B plus UV-A light synergism. No alteration of the synergistic response is seen in the presence or absence of ICX1. This leads to the conclusion that the negative regulation of ICX1 is removed in the synergistic response as discussed in Section 3.10 or that it is too small to be seen against the large background of *CHS* expression resulting from a UV-B plus blue or UV-B plus UV-A treatment.

6.2.1 What is ICX1?

Definite explanations of what ICX1 is will have to await mapping and sequence analysis. Guesses, albeit educated, can only be made prior to this. The mutation in *ICX1* affects a number of aspects of *Arabidopsis* growth and regulation. To recapitulate the visible phenotype; the *icx1* mutant is altered in leaf shape, trichome number, epidermal cell division/expansion, root hair spacing, seed mucilage production (Jackson *et al.*, 1995) and additionally is somewhat shorter than the wild type, with reduced root length (B. A. Brown, J. A. Jackson and G. I. Jenkins, unpublished). As mentioned previously, while altered in similar characteristics to *ttg*, *icx1* is not allelic to it (Section 1.8.2). The *TTG* gene has recently been identified as encoding a novel protein containing WD40 repeats (Walker *et al.*, 1999).

An increasing number of mutants have been isolated which are altered in anthocyanin accumulation. Some of these mutants have been found to be altered in sequences in petunia encoding bHLH (*an1*, de Vetten *et al.*, 1997), myb (*an2*, de Vetten *et al.*, 1997) and in *Arabidopsis* WD40 repeats (*ttg*, Walker *et al.*, 1999) and myc (*atmyc1*, Urao *et al.*, 1996) proteins. This opens up the possibilities for what ICX1 may be or may interact with.

The HY5 protein is required for *CHS* expression and is also involved in lateral root formation and elongation (Okada *et al.*, 1998), two factors that *icx1* is altered in. It may be that ICX1 and HY5 interact, or that the mutation in ICX1 allows easier interaction between HY5 and another binding factor or the *CHS* promoter itself.

While *icx1* does not display de-etiolation in the dark, there are some similarities between the phenotypes of *icx1* and *det2*, a mutant in the brassinolide biosynthetic pathway (Chory and Li, 1997). *det2* shows overexpression of *CHS* and it is a dwarf. Note that the hypocotyl extension of *icx1* was less than that of the wild type plants in both light and dark (Figure 3.8.7) though *icx1* is not a dwarf. Additionally another dwarf mutant, *sax1*, rescued by exogenous brassinosteroid application has been isolated which

displays altered leaf shape and root development (Ephritikhine *et al.*, 1999a). The *sax1* mutant has longer and denser root hairs and does not display de-etiolated responses in the dark. Interestingly, the *sax1* mutant is also mapped to the lower arm of Chromosome 1 (Ephritikhine *et al.*, 1999a) and is altered in an early step of a brassinosteroid synthesis pathway active in both light and dark (Ephritikhine *et al.*, 1999b). However, other aspects of the phenotype (the small, dark-green cotyledons and curled primary root) and the exact map position indicate that *sax1* is not allelic to *icx1*.

Possible predictions for ICX1 are that it is a transcription factor or is involved in the regulation of transcription factors, perhaps via phosphorylation. Phosphorylation of transcription factors by CK2 (casein kinase II) has been shown to regulate gene expression (Lee *et al.*, 1999). Reduction of the amount of active CK2 protein by expression of the antisense sequence of the α-subunit of CK2 resulted in increased expression of the light regulated genes, *CAB* and *rbcS* after red light treatment. The *CHS* expression level was increased in the dark (Lee *et al.*, 1999). This investigation of light mediated increase in gene expression level was limited to red light, which, as described here, and elsewhere (Kaiser *et al.*, 1995; Kubasek *et al.*, 1998) does not result in an increase in *CHS* expression in dark grown seedlings. The effect of light on *CHS* expression in combination with reduced levels of CK2 would be better investigated in blue or far red light treated dark grown seedlings. However the reduction of CK2 did affect *CHS* gene expression in the dark, which may point to a mode of regulation of *CHS* expression. If ICX1 is a transcription factor, perhaps the mutation in ICX1 seen in the *icx1* mutant impairs its ability to phosphorylate or be phosphorylated/dephosphorylated, and thus alters gene expression resulting in the pleiotropic phenotype.

An increasing number of proteins are being found to be transported into and out of the nucleus during light regulation of gene expression including COP1 (von Arnim and Deng, 1994), PHYB (Sakamoto and Nagatani, 1996; Yamaguchi *et al.*, 1999), and CPRF2 (Kircher *et al.*, 1999). ICX1 may be involved

in light regulation via physical regulation of the localisation of the light signalling components and also in the non-light regulated aspects of the phenotype.

6.3 Phytochrome Regulation of *CHS* Expression in UV light

The plants used to investigate the involvement of phytochrome in the UV light regulation of *CHS* were three week old plants which had been grown in constant low white light. It is therefore unsurprising that responses were seen in the *phyB* mutants rather than in *phyA*. Light labile phytochrome A would be present in very low amounts in the plants investigated. The use of the *phyA phyB* double mutants in the studies did however, ensure that there were no effects of PHYA or PHYB masking the role of the other phytochromes. Phytochromes C, D and E were not investigated, and therefore involvement of these phytochromes in the response of the *phyA* and *phyB* mutants cannot be ruled out.

As reported in Sections 4.3 and 4.4, phytochrome plays a role in regulation of *CHS* expression in response to both UV-A and UV-B light in three week old light grown *Arabidopsis* plants. In *Arabidopsis* a number of studies have indicated a role for interaction between cryptochrome and phytochrome in anthocyanin accumulation (discussed in Section 1.6). There have also been reports that cryptochrome requires phytochrome for full activity (Ahmad and Cashmore, 1997; Ahmad *et al.*, 1998b). Hypocotyl elongation inhibition is somewhat reduced if seedlings are given a pulse of far red light immediately after a blue light treatment (Casal and Bocalandro, 1995). Investigation of phytochrome interaction with cryptochrome in UV-A light absorption would be expected to parallel that seen in blue light as *CHS* expression in UV-A is almost entirely mediated by cryptochrome 1 (Fuglevand *et al.*, 1996). In the *phyB* single and the *phyA phyB* double mutants, *CHS* expression in UV-A light is reduced, indicating a role for phytochrome in regulating *CHS* expression. This result is opposite to the *CHS-GUS* expression reported by Batschauer

(1996) in UV-A light in *phyA*, *phyB* single and double mutant seedlings, though comparable to that reported by Neff and Chory (1998) and by Poppe *et al.* (1998) in blue light mediated anthocyanin accumulation in seedlings.

In some varieties of maize, red light increased the UV-B induced anthocyanin accumulation (Beggs and Wellman, 1994). In *Arabidopsis* investigated here, the increase in *CHS* expression seen in UV-B light in plants lacking phytochrome B implies there is an involvement of phytochrome B in UV-B light signal perception or signal transduction. That, in the absence of a photoreceptor we see an increase in the amount of *CHS* expression, may be due to phytochrome B signal transduction resulting in a negative regulation of *CHS* expression in the wild type plants. PHYB and a UV-B photoreceptor may have antagonistic roles in regulating *CHS* expression in UV-B light. Similar antagonistic roles of photoreceptors can be seen, for example, in the regulation of flowering time via the *CONSTANS* gene by PHYB and CRY2 (Guo *et al.*, 1998). Alternatively, the effect of the removal of PHYB may be causing multiple alterations in the photoreceptor composition, resulting in a larger pool of UV-B photoreceptors than is normally seen in the wild type plants.

A role for phytochrome in UV-B light responses has been intimated previously. Kim *et al.* (1998) report that their findings indicate that phytochrome plays a role in the regulation of photomorphogenesis in low fluence UV-B light. This investigation was carried out using 2 day old dark grown seedlings. UV-B treated seedlings were found to show inhibition of hypocotyl extension. This inhibition was absent in *phyA phyB* double null mutant seedlings. This indicates a role for UV-B mediating photomorphogenesis either via PHYA or PHYB in low fluence UV-B or for the requirement of phytochrome for UV-B photoreceptor regulation of photomorphogenesis.

In the experiments described here using mature *Arabidopsis* plants, the absence of both PHYA and PHYB results in an increase in UV-B light mediated expression of *CHS*. It is likely, given the developmental alterations an

Arabidopsis plant makes during it's life cycle, that a different effect is seen in dark grown *Arabidopsis* seedlings than in mature plants. The phytochromes show altered ability to mediate *CHS* expression (in far red light at least) as the plant matures (see Sections 1.9 and 3.3).

It could be argued that at the level of UV-B light used in this study we see an increase in *CHS* expression as a result of DNA damage providing a signal. However the fluence rate used here is equivalent to that in natural daylight. The explanation of DNA damage products acting as messengers does not explain how the removal of phytochrome B results in an increase in *CHS* expression above that of the wild type. The only way to explain this would be that phytochrome again acts in some way to dampen the signal, resulting in a lower level of *CHS* expression in the wild type plants.

Phytochrome B has different effects on regulation of *CHS* expression in different light qualities. PHYB acts to increase *CHS* expression in UV-A light and to decrease it in UV-B light. This may be (as suggested in Section 4.7) to optimise the UV protectant compounds produced in response to the light environment. In UV-A light flavonoids may be more effective and increased production of flavonoids is warranted. In UV-B light, sinapic acid esters appear to have a significant role (Landry *et al.*, 1995). To investigate whether phytochrome B plays a role in regulating the final components of the UV protective shield the relative levels of sinapic acid esters and flavonoids could be compared between UV-B irradiated *phyB* and wild type plants. The relative survival rates of *phyB* and wild type plants in UV-B light would indicate whether the absence of PHYB reduced the ability of plants to protect themselves against UV-B damage. In addition, the expression levels of *PAL* could be compared between the wild type and *phyB* plants. The enzyme PAL acts upstream from the point of divergence between flavonoids and sinapic acid esters. If this theory is correct, no difference would be expected to be seen in expression levels of *PAL* between *phyB* and wild type plants.

6.4 Screen for Mutants Altered in *CHS* Regulation

The initial work necessary for a screen to produce low *CHS* expressing mutants has been completed. The *CHS-CD* construct has been made and introduced into *Agrobacterium*. Vacuum infiltration of plants was completed and a number of putative transgenic plants have been isolated. After checking the progeny for kanamycin resistance and 5-FC sensitivity, the seed stock can be bulked up to be mutagenised. This sort of genetic approach is important as it is likely to provide information on positive factors required for *CHS* induction.

6.5 Possible Mechanisms of Light Signal Transduction

6.5.1 Nuclear Speckles

Kim *et al.* (1998) investigated the role of COP1 and DET1 in the UV-B signalling pathway. Kim *et al.* suggest that COP1 and DET1 act downstream from the UV-B signalling pathway from their study on low fluence UV-B light effects on photomorphogenesis. The interaction of COP1 and HY5 in the regulation of *CHS* expression in white light elucidated by Ang (1998) provides a further link in the chain. HY5 appears to be a transcription factor required for *CHS* expression. Work presented here on the involvement of HY5 in UV-A and UV-B regulated *CHS* expression supports that conclusion as in the absence of HY5, no UV-B light mediated *CHS* expression is seen (Figure 3.8.3 and 3.8.4). Yamaguchi *et al.* (1999) have reported that phytochrome B is translocated into the nucleus upon light exposure. This translocation was detected by PHYB-GFP transgene expression. Imaging of the GFP allowed observation of the formation of speckles of PHYB-GFP in the nucleus. This is reminiscent of the speckles formed by COP1 in the nucleus (Ang *et al.*, 1998). It is not yet known whether the speckles formed by COP1 are the same as those seen in the PHYB-GFP plant nuclei. Yamaguchi *et al.* suggest that the speckles indicate the site where nuclear factors interact. There is a case for this, especially with the recent work indicating that COP1 recruits HY5 into the speckles (Ang *et al.*, 1998), the requirement of HY5 to mediate *CHS* expression (Ang *et al.*, 1998) and the role of

PHYB in regulating *CHS* expression (Section 4.3 and 4.4). Thus, we have the indication that PHYB and HY5 are involved in regulating the same response, and interaction, perhaps in these nuclear speckles, may result in the expression levels obtained under different light conditions. This possibility would require HY5 retention in the speckles during the time when COP1 is being transported out of the nucleus and PHYB is being transported in. This is possible if COP1 acts to recruit HY5 into the nuclear speckles but is not the only binding partner able to keep it there. Alternatively the PHYB recruitment into the speckles may affect binding of other factors which can then leave the speckle to interact with HY5 or the *CHS* promoter.

6.5.2 The CPRF2 Model

Another way in which HY5 and phytochrome may interact to regulate *CHS* expression was suggested by Kircher *et al.* (1999). Kircher *et al.* showed that the common plant regulatory factor 2 (CPRF2) of parsley is translocated into the nucleus in light, predominantly in red light, and that this red light translocation can be partially reversed by a following far red light treatment. There is a family of CPRF's which contain bZIP domains, and act as transcription factors. CPRF2 was shown to bind to the G-box of the LRU of the parsley *CHS* promoter (Weisshaar *et al.*, 1991). From the work of Batschauer *et al.* (1996) the conclusion reached was that phytochrome is not involved in regulating *CHS* expression in *Arabidopsis*; therefore, Kircher *et al.* suggest that as the *CHS* gene is regulated by UV photoreceptors and not phytochrome that the CPRF2 target is unlikely to be the *CHS* promoter. If phytochromes do not regulate UV-A light mediated *CHS* expression in 2 day old seedlings, the same cannot be said of three week old plants.

As described above (Section 4.3), there is a role of phytochrome in UV-A light regulation of *CHS* expression in three week old plants. It is possible that CPRF2 binds to the G-box of the LRU and acts to increase *CHS* expression in response to blue and UV-A light. Some nuclear localisation of CPRF2 was seen

in both blue and UV-A light. The localisation of CPRF2 was not investigated in UV-B light in this study, so it is not known whether CPRF2 is nuclear localised in response to UV-B irradiation.

HY5 has not been shown to be transcriptionally active and may require a transcriptionally active bZIP for function (Ang *et al.*, 1998). Kircher *et al.* suggest that CPRF2 may function as a heterodimer with HY5. Given the additional effect of phytochrome in UV light regulation and the HY5 requirements for *CHS* expression in UV light, this CPRF2/HY5 interaction is more plausible.

6.5.3 Signal Specificity

With the idea that the COP9 signalosome forms a central component common in a number of regulatory pathways, where does the specificity of the response lie? Likewise, if all *CHS* expression requires HY5 (which is also involved in other responses, eg. hypocotyl elongation and lateral root formation, Koornneef *et al.*, 1980; Okada *et al.*, 1998) how are different rates and kinetics of response achieved? If the speckle theory that the transcriptional regulators in the nucleus are centralised in these speckles produces a specific response there must be something providing the specificity of the responses. It may be that while signal transduction pathways downstream from light receptors lead to COP1 nuclear exclusion and removal of HY5 repression, a second signal which provides for specificity is also produced. Since HY5 is not sufficient for *CHS* expression, requiring the presence of a transcriptionally active partner for activity (Ang *et al.*, 1998), a level of regulatory specificity for *CHS* expression in blue/UV light may be in the nuclear localisation of the transcriptionally active factor which can interact with both HY5 and the *CHS* promoter. It is possible that there are a number of bZIPs, MYBs and MYCs which can interact with HY5 and the *CHS* promoter. The light responsive unit which Hartmann *et al.* (1998) identified by promoter deletion-GUS assay was the same for both UV-B and UV-A light regulation in the *Arabidopsis CHS*

promoter. The level of *CHS* expression seen in response to UV-A or UV-B light is not the same (see Section 3.2). I suggest that the bZIP/MYB/MYC (or a combination) factor which is translocated into the nucleus is different in response to UV-B light than to UV-A light and it is this difference which results in the altered expression kinetics in UV-A and UV-B. It may even be that PHYB can act to regulate binding factors in some way, which results in the differences in expression seen in the *phyB* mutant in either UV-A or UV-B light.

Harter *et al.* (1994) reported that protein synthesis was involved in activation and transport into the nucleus of transcription factors which can interact with box II in the LRU1 of the *CHS* promoter of parsley. There is also a requirement for protein synthesis for *CHS* induction in both UV-A/blue and UV-B light (Christie and Jenkins, 1996). It may be that the protein synthesis requirement in *CHS* induction is for UV-A or UV-B specific transcription factor activity or localisation.

6.6 Further work

The work presented in this study allows a number of investigative avenues to be followed. The characterisation of the *icx1* mutant has provided a basis for the involvement of ICX1 in *CHS* regulation. In addition, a role for phytochrome in UV light regulation of *CHS* gene expression in *Arabidopsis* has been identified.

The apparent absolute requirement for HY5 in *CHS* expression could be addressed further. What is the involvement of HY5 in non-light mediated signals, for example, cold or cytokinin increases in *CHS* expression?

The level of sucrose present in a plant appears to be correlated with the level of *CHS* expression upon light induction (Sections 3.8 and 3.10). This apparent requirement for sucrose could be investigated in seedlings grown on +/– sucrose, in the dark, then exposed to a non-photosynthetically inductive light source, such as UV-B. A sucrose requirement will be indicated by observing whether *CHS* is expressed in seedlings grown on both plates or only

+S plates. To clarify whether the *CHS* expression increase seen in red followed by UV-A light is a result of phytochromes C, D, E or sucrose/metabolic intermediates, the use of low fluence red light pulses followed by far red light pulses, or *vice versa,* prior to UV-A exposure could be utilised. If the classic phytochrome reversibility is observed, it is likely that the response is due to phytochrome involvement. In a complementary experiment, plants could be illuminated with a photosynthetically active light (such as a red/far red combination) which does not alter the phytochrome Pr:Pfr ratio, and if an increase is seen in response to this pretreatment the involvement of sucrose and or metabolic intermediates would be indicated.

An obvious next step, having provided the basis of a screen, would be to utilise the CD screen to identify positive regulators of *CHS* expression. The screen could be easily modified to search for mutants specifically altered in responses downstream from a specific photoreceptor and, with luck, could even isolate a UV-B photoreceptor. By completing the screens in specific light qualities, further elucidation of the light signalling network can be achieved.

Appendix I

Restriction Enzyme Digests Described in Chapter 5

pMSCD 100% *Sma*I digest	**pMSCD partial *Bam*HI digest**
25 µl pMSCD	38 µl pMSCD *Sma*I digest
3 µl *Sma*I	0.1 µl *Bam*HI (1 U)
4 µl Buffer J	8µl Buffer E
4 µl BSA	4 µl BSA
4 µl dH_2O	30 µl dH_2O
Total volume 40 µl	Total Volume 80 µl

Subsequent to treatment for Klenow 5' Fill In

18 µl pMSCD (minus the MS promoter)

1 µl *Hin*dIII

3 µl Buffer E

3 µl BSA

5 µl dH_2O

Total Volume 30 µl

p459 sequential digest

30 µl p459	40 µl p459 *Nae*I digest
3 µl *Nae*I	3 µl *Hin*dIII
4.5 µl Buffer A	8µl Buffer E
4.5 µl BSA	3.5 µl BSA
3 µl dH_2O	25.5 µl dH_2O
Total volume 45 µl	Total Volume 80 µl

Appendix II

CHS coding region included in the *CHS-CD* cassette

```
*ATG AGA TCA GAC AGG CTC AGA GAG CTG ATG GAC ctg cag GCC `GCC
```

CHS start site at 2596 (*)

*Nae*I site cuts at 2554 (`)

Primer used to sequence pCHSCD

```
CACAACAGCAACATCAAACT
```

Expected Sequence from the *CHS* start site to the CD start site

```
   ATGGTGATGGCTGGTGCTTCTTCTTTGGATGAGATCAGACAGGCTCAGAGAGCTGATGGA
 1 ---------+---------+---------+---------+---------+---------+60
   TACCACTACCGACCACGAAGAAGAAACCTACTCTAGTCTGTCCGAGTCTCTCGACTACCT
   M  V  M  A  G  A  S  S  L  D  E  I  R  Q  A  Q  R  A  D  G

   CCTGCAGGCCGATCCGCGATG
61 ---------+---------+- 81
   GGACGTCCGGCTAGGCGCTAC
   P  A  G  R  S  A  M
```

Sequence obtained from pCHSCD

The sequence indicates that the start sites of *CHS* and *CD* are in frame, nucleotide 1 of pCHSCD correlates with number 41 of the expected sequence.

```
   GGCTCAGAGAGCTGATGGACCTGCAGGCCGATCCGCGATGTCGAATAACGCTTTACAAAC
 1 ---------+---------+---------+---------+---------+---------+60
   CCGAGTCTCTCGACTACCTGGACGTCCGGCTAGGCGCTACAGCTTATTGCGAAATGTTTG
    A  Q  R  A  D  G  P  A  G  R  S  A  M  S  N  N  A  L  Q  T

   AATTATTAACgCCCGGTTACCAGGCGAAGAGGGGCTGTGGCAGATTCATCGCAGGACGGA
61 ---------+---------+---------+---------+---------+---------+120
   TTAATAATTGcGGGCCAATGGTCCGCTTCTCCCCGACACCGTCTAAGTAGCGTCCTGCCT
    I  I  N  A  R  L  P  G  E  E  G  L  W  Q  I  H  R  R  T  E

   AAAATCAGCGCCATTGATGCGCGAATCCGGCGTGATGCC
121 ---------+---------+---------+--------- 159
   TTTTAGTCGCGGTAACTACGCGCTTAGGCCGCACTACGG
    K  S  A  P  L  M  R  E  S  G  V  M
```

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