GENES REGULATED BY R:FR RATIO AND THE CIRCADIAN CLOCK IN *ARABIDOPSIS THALIANA*

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by
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Abstract

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Lisa F Doyle

The shade avoidance response is of major adaptive significance to plants. Angiosperms in particular have developed a wide range of strategies to avoid shade. In canopy shade conditions, the ratio of red to far-red light wavelengths decreases (low R:FR ratio) this acts as a signal to instruct plants that they are in the proximity of neighbouring flora. A decrease in R:FR ratio is perceived through the phytochrome system, which results in onset of several developmental responses; such as elongation growth; that help promote escape from shade. Although much is known of the role phytochromes play in light perception and the concomitant physiological responses triggered by a decrease in the R:FR ratio, little is known of the downstream molecular components that bring about these changes.

This study focuses on a small number of genes (*DIN2*, *ENDO1* and *XTH15*) that whilst known to be R:FR ratio regulated, have as yet unknown roles during shade avoidance. This work demonstrates that all three genes respond with extreme rapidity and reversibility to changes in light quality conditions. The role phytochromes play in regulation of these genes is investigated. In addition a number of light regulated transcription factors, downstream of the phytochromes, are identified as intermediate regulators of these genes.

As it has recently come to light that a number of R:FR ratio regulated genes are also under the control of the circadian clock the ability of this signalling pathway to influence expression of these genes was also investigated. Molecular analysis revealed that the circadian clock modulates two of the three genes examined within this study.

Finally, systematic physiological characterisation of mutant and over-expressing lines was performed with an aim to determine the biological function of these genes during shade avoidance. Additionally, this study reveals that one of the genes studied; *XTH15*; not only exhibits R:FR ratio regulation of expression, but also blue light mediated transcriptional responses. This raises the possibility that R:FR ratio and blue light signaling pathways converge. Preliminary data suggests that *XTH15* could represent a point of cross over with the phytochromes and the cryptochromes.

This work, which characterises the response and potential role of three R:FR regulated genes downstream of the phytochromes, provides important and novel contributions to our understanding of the environmental regulation of plant development.

Abbreviations used in this study

B: Blue light

CAB: Chlorophyll a/b binding protein

CaMV: Cauliflower mosaic virus

d: day

FR: Far-red light

GFP: Green Fluorescent Protein

GUS: ß-glucuronidase

h: Hour

HIR: High Irradiance Response

kDa: Kilodalton

LD: Long Day

LFR: Low Fluence Response

LUC: Luciferase reporter gene

M: Molar

min: Minute

mol: Mole

OX: Over-expresser

PCR: Polymerase Chain Reaction

QPCR: Quantitative RT-PCR

R: Red light

R:FR ratio: Red: Far-Red ratio

SD: Short Day

sec: Second

VLFR: Very Low Fluence Response

WL: White light

WT: Wild-type

YFP: Yellow Fluorescent Protein

Contents

1. Introduction

The overall aim of this study is to advance understanding of the multifactorial endogenous and exogenous signals that underpin shade avoidance behaviour in *Arabidopsis thaliana*. In particular, this study focuses on genes that are putatively regulated by the phytochromes, a family of proteins known to be key mediators of the shade avoidance response. Although many studies have focused on the physiological effects that phytochromes induce under specific light conditions, most notably decreases in R:FR ratio light, little is known of the downstream molecular components underpinning these responses. This study seeks to define some of the elements within this pathway.

Throughout my postgraduate studies I have focused primarily on three genes; *DIN2* (*DARK INDUCIBLE 2*), *ENDO1* (*ENDOCHITINASE LIKE GENE 1*) and *XTH15* (*XYLOGLUCAN ENDOTRANSGLUCOSYLASE/ HYDROLASE 15*); that may participate in shade avoidance signalling with an aim to understand how they are integrated into this pathway. The impetus to study these particular genes arose from preliminary data demonstrating that their expression is influenced by a decrease in low R:FR ratio light (Whitelam pers com, 2003). I have sought to understand how expression of these genes is affected by different light quality conditions and how members of the phytochrome family regulate these changes. In addition, I have examined the role of several candidate transcription factors, known to be part of the shade avoidance pathway, in regulating expression of these genes. Subsequently, through mutant and transgenic analysis, I have attempted to establish how these genes contribute to the shade avoidance phenotype. Finally, in light of the recent reports demonstrating that light regulated genes are also subject to circadian control, I have

examined whether this intrinsic biological mechanism affects expression of *DIN2*, *ENDO1* and *XTH15*.

In view of the aims above the introductory chapter is organised into the following sections:

- The plant photoreceptors, the phytochrome and cryptochrome families in particular, and their structure and regulation under different light conditions
- The shade avoidance response, the role of different phytochromes in shade avoidance, phenotypic responses to reduced R:FR ratio light
- The interaction between phytochromes and cryptochromes during shade avoidance
- Transcriptional regulation of shade avoidance genes
- The circadian clock: how it operates and how it gates R:FR responses to light.

Plants have evolved an ability to optimise their growth to suit the environment. Such phenotypic responses are induced by spatio-temporal variation in environmental factors, examples being the availability of light, water and nutrients. As phototrophic organisms, light is one of the major factors of these developmental adaptations. Plants have evolved a series of photoreceptors, which enable detection of light quality, quantity and direction. The perception of these light signals is crucial to the appropriate acclimation of plants to their natural environment. Additionally, through interactions with the central circadian oscillator, light signals enable plants to monitor photoperiod, predict dawn/ dusk and adjust their developmental pattern, a process known as "photomorphogenesis". The effective perception, transduction and interpretation of light signals through photoreceptor signalling is essential to an individual plant's success.

The photoreceptors can be divided into four groups; the phytochromes, detectors of red (R) and far-red (FR) light; the cryptochromes detectors of blue (B) and UV-A; the UV-B detectors; and the phototropins which detect blue and UV-A. Our current understanding of the role each of these photoreceptors plays in plant growth and development, from germination to seed maturation, is reviewed below.

1.1. Photoreceptors

1.1.2. Phytochromes

The phytochromes play critical roles in monitoring light quantity, quality and periodicity in higher plants. Phytochromes also monitor the direction of light in lower plants. These proteins relay photo-sensory information to a number of signalling pathways involved in regulation of plant growth and development. Phytochromes are reversibly photochromic photoreceptors: Light modifies the interaction between chromophore and polypeptide components of the phytochromes, enabling them to assume two spectrally distinct forms. These two, relatively stable isoforms, are a R light-absorbing form, Pr; with an absorption maximum at about 660 nm; and a FR light-absorbing form, Pfr; with an absorption maximum at about 730 nm. Phytochrome is synthesised as Pr, which is biologically inactive with regard to phytochrome mediated-responses. The absorption spectra of Pr and Pfr show relatively large overlap in the visible light spectrum. Biological activity is acquired upon phototransformation to the Pfr isomer (Kendrick and Kronenberg, 1994).

1.1.3. Phytochrome structure and regulation

Three major phytochromes exist in angiosperms, phyA phyB and phyC. In the model species *Arabidopsis thaliana*, there are five apophytochrome genes *PHYA-PHYE* (Clack *et al,* 1994). These five discrete apophytochrome-encoding genes have been isolated and sequenced (Matthews and Sharrock, 1997). Sequencing information suggests that phytochromes B and D are closely related, sharing 80% sequence homology to one another. These phytochromes are more related to PHYE (55% homology) than to PHYA and PHYC (47% homology) and are therefore thought to have formed a more recent subgroup (Goosey *et al*, 1997). An early duplication event is likely to have separated *PHYA* and *PHYC* from *PHYB, PHYD* and *PHYE*. Plant phytochromes have remarkably highly conserved primary and secondary structures and their coding sequences and, with the exception of PHYC, are interrupted by three introns positioned at conserved sites (Sharrock and Matthews, 2006).

Phytochrome molecules are homodimers, with each polypeptide monomer being *ca.*125 kDa in mass. Each of the monomers carries a single, covalently-linked linear tetrapyrole chromophore (phytochromobilin PΦB) attached via a thioether bond to a conserved cysteine residue in the N-terminal domain of the protein (Furuya and Song, 1994). Both plant phytochromes and bacteriophytochromes share a common architecture consisting of three conserved domains (termed P2 or PAS domain, P3 or GAF domain and P4 or PHY domain) and a C-terminal domain (histidine kinase-related domain), which is responsible for non-covalent dimerization of the molecule. In plants this region may also have a role in kinase-mediated signalling following photoconversion of phytochrome to its active conformation (Al-Sady *et al*, 006), although recent studies suggest this domain is not necessary for transduction of light signals (Matsushita *et al*, 2003). Plant phytochromes have an additional N-terminal

extension termed P1, which is known to inhibit dark reversion, discussed in detail later in this chapter (Vierstra, 1993), and two additional PAS domains, which contain nuclear localisation motifs (Chen *et al*, 2005).

Light induced photoconversion between Pr and Pfr isoforms occurs through Z-E isomerisation about the C15-C16 double bond of phytochromobilin. The photosensory function of the molecule is based on its capacity for reversible interconversion between the R light-absorbing form Pr and the FR-absorbing Pfr form mediated by the absorption of light. The FR light-absorbing form of phytochrome (Pfr) is considered as biologically active as it is in this conformation that physiological responses are initiated. The absorption spectra of both Pr and Pfr overlap considerably throughout the visible light spectrum. Therefore, *in vivo* the two phytochrome isoforms will exist in equilibrium under almost all irradiation conditions. Both Pr and Pfr also absorb UV-B and B, a phenomenon that is thought to be physiologically relevant as phytochromes assist the phototropins and the cryptochromes in mediating B light responses (Franklin *et al*, 2003). Pfr formation (signal perception) initiates an intracellular transduction process that results in altered expression of genes mediating the photomorphogenic response, whereas re-conversion to Pr can abrogate this process.

1.1.4. Light-induced nuclear import of phytochrome molecules

Recent studies have provided evidence that phytochrome molecules translocate from the cytoplasm to the nucleus following photoactivation. Data from several laboratories using ß-glucuronidase (GUS), phy-green fluorescent protein (GFP) fusion and immunocytochemical localization procedures indicate that phytochromes exist in the cytoplasm in the inactive Pr isoform (Sakamoto and Nagatani, 1996; Kircher *et al*,

1999). Following light-induced photoconversion to biologically active Pfr conformers, these molecules migrate from the cytoplasm to the nucleus.

Proteomic Western blot studies examining the effects of light quality on phytochrome localisation in isolated nuclei of light grown plant leaves have further shown that the majority of PHYB is located in the nucleus (Sakamoto and Nagatani, 1996), whilst dark treated plants have greatly reduced nuclear PHYB levels. This reduction is markedly accelerated if plants are exposed to FR light prior to dark rearing (Sakamoto and Nagatani, 1996). In support of this, when PHYB-GFP fusion constructs under the control of the 35S promoter are transformed into plants that are subsequently grown in darkness, the majority of cells show GFP expression concentrated in the cytoplasm with only a few cells displaying (weak) nuclear presence. In contrast, when the same plants are treated with WL, GFP translocates into nuclei. Maximum nuclear accumulation occurs within approximately 6 h (Kircher *et al*, 1999; Yamaguchi *et a*., 1999).

Once in the nucleus, PHYB appears to aggregate into speckles (Kircher and Nagy, 2002; Nagy *et al*, 2000; Gil *et al*, 2000). PhyB GFP fusion experiments have shown that nuclear speckles form after 1 h of continuous R light, whereas 3 h of FR fails to induce this phenomenon. In addition a 3 h pulse of blue light induces only minimal speckling. Once plants are returned to darkness, nuclear speckles decline, although this effect is not immediate (half life approximately 3 h). More recently it has been shown that plants that are radiated by R light prior to being moved to darkness and subsequently exposed to a pulse of R light for 15 mins have elevated levels of PHYB, similar to those observed after 3 h of R radiation in dark grown seedlings. A FR pulse after the R pulse completely reverses this effect. It is now

known that in fact PHYB nuclear speckles can be seen after only 5 seconds of R irradiation (Kircher and Nagy, 2002, Nagy *et al*, 2000; Gil *et al*, 2000).

The speckle-like aggregation of PHYB within the nucleus appears to depend upon the C terminal domain of the protein, as mutation of specific residues in this region cause loss of nuclear speckling (Ni *et al*, 1999). Furthermore, when the C terminus and the N terminus of PHYB are fused to GFP and expressed under the control of the 35S promoter, the C terminal construct localises in the nucleus whereas the N terminal construct disperses evenly throughout the cytoplasm and the nucleus (Ni *et al*, 1999).

PHYA has been found to exhibit similar cellular localisation behaviour. When seedlings containing PHYA-GFP transgenes are dark-grown, no nuclear GFP can be observed by day 6 in development. However, short pulses (5 mins) of R, FR or B light cause translocation of PHYA-GFP to the nucleus and speckle formation (Kim *et al*, 2000). Interestingly, a 5 min pulse of FR light causes cytosolic speckles and then subsequent nuclear import. Prolonged B or FR also causes cytosolic speckling however, prolonged R leads to a loss of GFP expression in both the cytoplasm and the nucleus. This work indicates that both FR HIR and VLFR can result in nuclear localisation (Kim *et al*, 2000).

It is now known that WL causes nuclear accumulation of PHYA speckles. In seedlings expressing PHYA-GFP fusion constructs, a peak in nuclear staining is reached after 10 mins of WL exposure. However, prolonged WL causes a rapid decline in expression of GFP, thus supporting the idea that prolonged irradiation with certain wavelengths of light induces PHYA degradation. If seedlings are irradiated with FR, similar staining can be seen with a peak in nuclear staining occurring after 2 h of light treatment (Kircher and Nagy, 2002).

The nuclear localisation of phytochrome C, D and E has also been analysed in plants transformed with phytochrome genes fused to the 35S promoter and a GFP coding sequence. For each of these transgenes GFP expression was found to occur as intense and diffuse nuclear speckles when transgenic seedlings were dark grown. Exposing seedlings to WL caused formation of nuclear speckles of PHYC and PHYE after 2 h, which reached maximum levels at around 6 h. WL did cause the formation of some nuclear speckles of PHYD but levels remained low and variable (Kircher *et al*, 2002).

The precise functional role of phytochrome-containing nuclear speckles is a subject of considerable debate. It is clear that activation of phytochromes controls expression of some 2,500 Arabidopsis genes under sustained light exposure conditions **(**Castillon, 2007). However, the significance of nuclear speckling during this process is unknown. One hypothesis is that these speckles represent 'transcriptosome' complexes, which regulate gene expression at specific locations in the genome (see diagram 1.1.4 below). Alternately, they may simply represent sites of phytochrome degradation (Nagy *et al*, 2000; Tepperman *et al*, 2001).

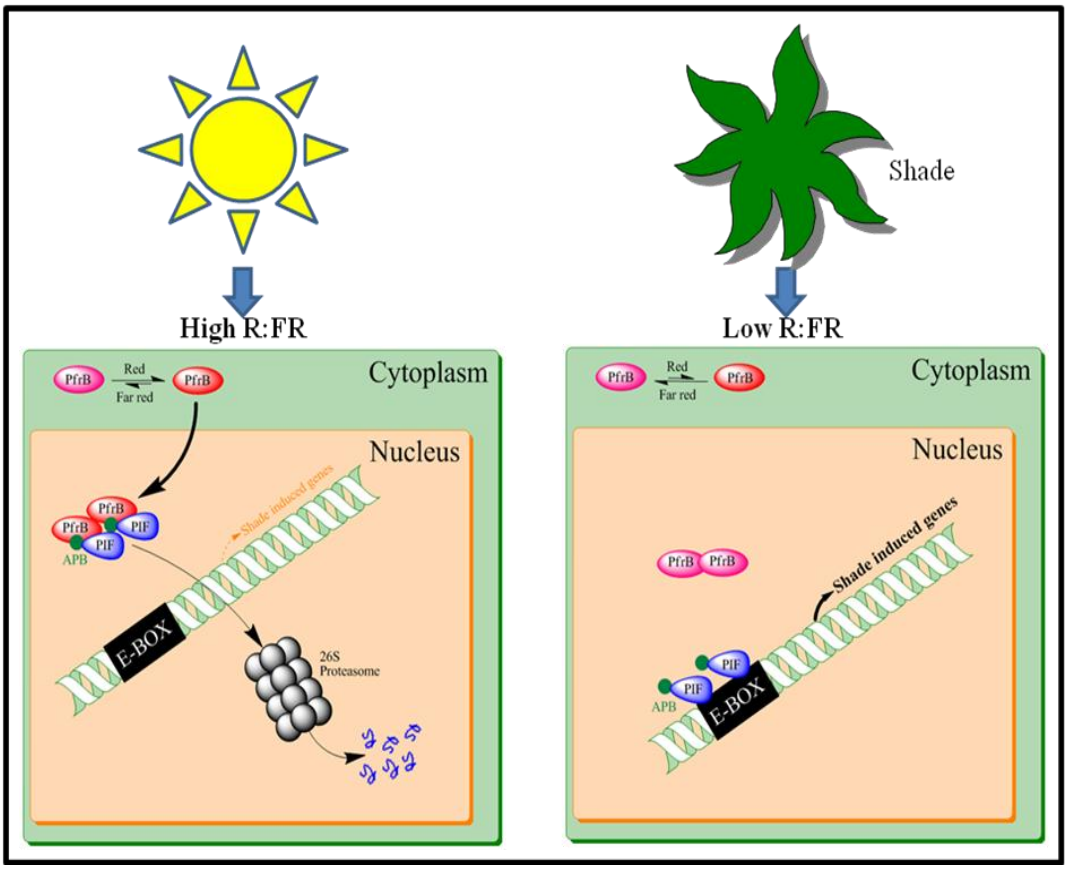


Diagram 1.1.4. Simplified model for the control of shade avoidance through phyB and the PIFs (PHYTOCHROME INTERACTING FACTORS). In sunlight, phyB is mainly in the Pfr form whereby it accumulates in the nucleus and interacts with the PIFs via the APB (ACTIVE PHYTOCHROME BINDING DOMAIN). This interaction targets PIF proteins for degradation through the proteasome pathway, which leads to the deactivation of genes induced by low R:FR ratio (shade conditions). Under vegetation canopy light is enriched in the FR region which causes phyB to be converted into the Pr form which exits the nucleus. In the absence of PhyB interaction, PIF proteins rapidly re-accumulate and promote expression of shade avoidance genes (such as *PIL1* and *ATHB2*).

1.1.5. Degradation

Regulation of plant cellular components is not only controlled by transcriptional and translational mechanisms but also by targeted protein degradation. The targeting of R:FR ratio regulated proteins for ubiquitination and proteasome degradation is influenced by light. COP1 (CONSTITUTIVELY PHOTOMORPHOGENIC 1), a ring motif-containing E3 ubiquitin protein ligase, is a key component in this process that acts downstream from the phytochrome and cryptochrome proteins (Ang and Deng, 1994). When seedlings are irradiated with light, COP1 translocates from the nucleus to the cytoplasm. This in turn allows known positive regulators of photomorphogenesis, such as the transcriptional regulator HY5 to accumulate, thereby permitting photomorphogenesis to occur (Wang *et al*, 2001). Phytochrome A has been shown to be ubiquitinated by COP1 *in vitro* (Seo *et al*, 2004). However, light-induced destruction of phyA Pfr persists in *cop1* mutant plants, suggesting that additional E2/E3 ubiquitin ligases regulate phyA Pfr degradation. This is perhaps not surprising as the covalent binding of multiple ubiquitins is required for ubiquitin-protein complex formation and subsequent commitment to degradation via the 26S proteasome complex (Ciechanover, 1994; Vierstra, 1996).

The 26S proteasome is a highly conserved protein degradation complex in eukaryotes. This complex is comprised three particles; a core 20S catalytic particle and two 19S regulatory particles. One 19S particle recognises and removes polyubiquitin chains on target proteins before unfolding them, a process facilitates efficient delivery into the 20S catalytic core. Once in the core, proteins are degraded through catalytic cleavage of polypeptide bonds (Small and Vierstra, 2004). These are then discharged through the other 19S particle.

1.1.6. Phytochrome response modes

1.1.6.1. The Low Fluence Response modes (LFR)

This response is characterised by the classic R/FR photochromic reversibility of phytochrome, observed by Borthwick *et al*, (1954). The LFR obeys the Bunsen-Roscoe reciprocity law; i.e. the response is proportional to the quantity of photoproduct. This can be produced by either low photon irradiance for long periods, or by high photon irradiance for short periods. The LFR is saturated at low fluences, the response being activated from 1 μmol m^{-2} s^{-1} R light. Increasing dark intervals between R and FR lead to an escape from reversibility, as enough time elapses in this dark period for Pfr action.

1.1.6.2. The Very Low Fluence Response (VLFR)

The very low fluence response is a phyA-mediated response observed in dark-grown seedlings. Initiation of this extremely sensitive response requires only 0.1 μmol m^{-2} s^{-1} or less of R light. VLFR can only be observed in dark-grown imbibed seed or seedlings in which only Pr is present. This response shows no R:FR reversibility as it can be mediated by FR, which requires very few molecules of Pfr for saturation. The Bunsen-Roscoe law of reciprocity is still obeyed.

1.1.6.3. The High Irradiance Response (HIR)

The HIR is both a phyA- and a phyB- mediated response and is dependent on fluence rate and prolonged periods of light exposure. The HIR operates in R, FR, B, UV-A and UV-B. The extent of the HIR is a function of the fluence rate, exposure time and wavelength. Although prolonged exposures are required for the function of HIR, continuous exposure is not necessary, as cyclic irradiations of equal fluence rate over

the same period as continuous irradiation is sufficient to mediate the same response providing that the dark periods are limited in duration. Examples of HIR include anthocyanin synthesis and seedling survival in shade conditions whereby FR-HIR acts to prevent excessive elongation growth of the hypocotyls. Furthermore, there is an interaction between the HIR and LFR. The effect of the R/FR reversible LFR in etiolated seedlings can be enhanced by prolonged light treatments with respect to inhibition of hypocotyl elongation, cotyledon expansion and anthocyanin production (Mancinelli, 1994). De-etiolation responses such as inhibition of hypocotyl elongation, hook opening and cotyledon expansion are mediated by each of the different phytochrome response modes.

1.1.7. Dark Reversion

The Pfr state of phytochrome is thermally unstable in most phytochromes, with restoration of Pr state over time in a process known as dark reversion. This mechanism makes an important contribution to the balance between Pr and Pfr forms (Mancinelli, 1994). Dark reversion is thought to be the mechanism underlying phytochrome inactivation (Elich *et al*, 1997).

1.1.8. Blue light photoreceptors

In 1881, Darwin first documented B light-induced plant phototropic responses (Darwin, 1881). However, it was only recently that B light receptor mediated phototropism, and other photoresponses, has been fully investigated.

Cryptochromes are found not only in plants, but also animals and bacteria. In plants the cryptochromes work together with phytochromes to regulate photomorphogenesis and recent studies show that cryptochromes also play a role in

circadian rhythms. Finally, cryptochromes have DNA photolyase domains which implicates them in the repair of blue/ UV-A-dependent DNA damage (Lin, 2002).

In Arabidopsis, sensitivity to B wavebands is conferred primarily by phototropins (phot1 and phot2) and cryptochromes (cry1 and cry2). Cryptochrome 1 and 2 differ in light-lability and fluence rate specificity: cry1 is light-stable and acts at higher fluence rates > 10 μmol m^{-2} s^{-1} of B whereas there is rapid decline of cry2 levels occurring at higher photon irradiances of B light (Lin *et al*, 1998).

A member of the Cryptochrome DASH (DROSOPHILA, ARABIDOPSIS, SYNECHOCYSTIS and HOMO) family (cry3) has been identified. Whilst the cry3 protein shows no photolyase activity, it does bind FAD (FLAVIN ADENINE DINUCLEOTIDE). Photolyase enzymes are involved in repairing UV light induced pyrimidine dimers in DNA cry3 is thought to act as a photoreceptor, although its exact function has not yet been discovered (Kleine *et al*, 2003).

A number of other gene products have now been implicated in blue light signalling. For example, FKF1 (FLAVIN-BINDING KELCH REPEAT F-BOX 1), a protein known to be involved in flowering response regulation is likely to encode a B light photoreceptor. Two other members within this family *ZTL* (*ZEITLEUPE/ LOV-KELCH PROTEIN 1/ ADAGIO 1*) and *LKP2* (*LOV-KELCH PROTEIN 2/ ADAGIO 2*) are also thought to transduce B light signals and play a role in the circadian clock (Imaizumi *et al*, 2003; Somers *et al*, 2000; Schultz *et al*, 2001).

1.1.12. Cryptochrome structure and regulation

Most plant species studied have multiple photolyase and cryptochrome members. All members of the cryptochrome and photolyse family share an amino terminal photolyase-related (PHR) domain and it is this domain that is responsible for

chromophore binding. Arabidopsis CRY1 and CRY2 are 58% identical in their PHR domains but share only 14% sequence similarity in their C-terminal domains. Tomato has at least three cryptochrome genes, *CRY1*a, *CRY1*b and *CRY2* (Perrotta *et al*, 2000; Perrotta *et al*, 2001). Interestingly, the amino acid sequences of tomato *CRY1* and *CRY2* are more similar to their Arabidopsis counterparts than to each other (Perrotta *et al*, 2000). The PHR domain of tomato CRY1 is 85% identical to the PHR domain of Arabidopsis CRY1 and only 63% related to tomato CRY2. This observation indicates the gene duplication event that resulted in plant *CRY1* and *CRY2* formation occurred at least 100 million years ago, before the divergence of the *Brassicaceae* (e.g. Arabidopsis) and the *Solanaceae* (e.g., tomato) (Perrotta *et al*, 2000).

Arabidopsis genes *CRY1* and *CRY2* show strong homology to one another and to bacterial DNA photolyase genes (Ahmad and Cashmore, 1993; Lin *et al*, 1998). Most plant cryptochromes are 70-80 kDa proteins with two domains, an N-terminal photolyase-related domain that shares sequence homology to DNA photolyases and a C-terminal extension that has no relation with photolyase. The PHR represents the chromophore-binding domain and the C-terminal extension is involved in nuclear/ cytosol trafficking and protein-protein interactions. DNA photolyases are flavoproteins that catalyse blue/ UV-A-dependent repair of DNA damage (Lin, 2002). All photolyases contain a flavin adenine dinucleotide chromophore and have either deazaflavin or pterin as a light-harvesting chromophore (Sancar, 2004). Almost all residues that are known to be important for chromophore binding in photolyases are conserved in cryptochromes whereas those regions critical for binding of DNA lesions and catalysis of DNA repair are not equally conserved in cryptochromes (Ahmad and Cashmore, 1993). However, cry1 does not have any detectable photolyase activity despite it containing FAD and a pterin (methenyltetrahydrofolate,

MTHF) chromophore (Malhotra *et al*, 1995). Arabidopsis cry1 and cry2 are nuclear proteins. Arabidopsis cryptochromes accumulate in the nucleus constitutively (cry2) or in dark conditions (cry1). The C-terminal of these proteins is sufficient for nuclear/cytoplasmic trafficking (Yang *et al*, 2000).

Despite a lack of overall structural similarity in the C-terminal extension of different cryptochromes, there are three conserved motifs. These three motifs and their linear order are well conserved in cryptochromes from Arabidopsis to *Physcomitrella*. The C-terminal extension containing these motifs is referred to as the DAS (DQXVP, ACIDIC and STAES) domain (Lin, 2002). Genetic studies indicate that the DAS region is important for cellular localisation, intermolecular interaction and physiological function of cryptochrome (Lin and Shalitin, 2003). The presence of these three motifs (from moss to angiosperms) in such an orderly arrangement suggests that the DAS domain may have existed in the ancestral cryptochrome.

As stated above, it has been suggested that Arabidopsis cry2 is constitutively imported into the nucleus in a manner that is independent of light treatment. This has been demonstrated in transgenic plants expressing fusion proteins of CRY2-GUS and CRY2-GFP which accumulate in the nucleus of transgenic plants grown in light and dark conditions (Mas *et al*, 2000). Cryptochrome 1, although imported into the nucleus in the dark, may either remain in the cytoplasm or become exported from the nucleus in response to light exposure. The expression of these genes is also spatially regulated. In GUS::CCT1 (CRY1 C-terminus) transgenic plants, fusion protein was mostly found in the nucleus in root hair cells of dark-grown transgenic plants whilst it was predominantly cytosolic in light-grown transgenic plants (Yang *et al*, 2000). Blue light has also been found to induce the interaction of phyB and cry2 in the formation of nuclear speckles. This was shown by fluorescent resonance energy transfer

microscopy using phyB-GFP and cry2-RFP constructs. The two constructs were also shown to interact in a light-dependent fashion (Mas *et al*, 2000).

1.1.13. Light regulation of cryptochrome expression

Arabidopsis CRY1 expression is not obviously affected by light whereas CRY2 is negatively regulated by blue light (Ahmad *et al*, 1998; Lin *et al*, 1998). When etiolated seedlings were exposed to 20-30 μmol m^{-2} s^{-1} blue light, the level of cry2 decreased to a new steady state within approximately 15 min (Shalitin *et al*, 2002). This decrease has been demonstrated to be the result of protein degradation. Both the PHR domain and the C-terminal extension appear to be important for B light-induced degradation of cry2. It is also thought that the cry2 protein is likely to mediate its own degradation, as its turnover is not affected by either phytochrome or *cry1* mutation (Lin *et al*, 1998; Yang *et al*, 2001).

1.1.14. Phototropins

The phototropins are the most recently characterised blue/ UV-A light absorbing photoreceptors in plants. Phototropins have been shown to control both growth along a specific trajectory in response to directional light (phototropism) and light-induced intracellular chloroplast movement (Sakai *et al*, 2001; Jarillo *et al*, 2001). The Arabidopsis phototropin family comprises two members; termed phot1 and phot2; which are highly homologous. The 'phot' proteins each have two distinct domains; a C-terminal serine/threonine kinase domain and an N-terminal region, the latter of which encodes two LOV (Light, Oxygen, Voltage) sub-domains. These sub-domains were originally found in light sensing, oxygen sensing and voltage-gated potassium

channel proteins (Huala *et al*, 1997). The LOV domain peptides have been shown to bind the chromophore flavin mononucleotide (FMN) (Christie *et al*, 1999; Sakai *et al*, 2001).

1.2. The natural light environment

It is thought that spectral variation from natural sunlight signals changes in plant physiological processes, thereby promoting adaptation and survival under modified light environments. Plants use the R/FR reversible activity of phytochromes to detect and measure light quality, which is essential in order for them to optimise their morphological form and photosynthetic activity in an ever changing environment. The natural light environment can be measured in relation to the phytochromes by calculating the ratio of the spectral photon irradiance (see below).

$$\text{R:FR ratio} = \frac{\text{Photon irradiance between 660 and 670 nm}}{\text{Photon irradiance between 725 and 735 nm}}$$

It is known that the R:FR ratio of daylight is approximately 1.15 and remains fairly constant even during cloud covering or haze conditions. The R:FR ratio in daylight decreases as dusk advances. This is due to the larger proportion of longer wavelengths from enhanced absorption and scattering within the atmosphere. At the end of the day, there is relatively little R in the light spectrum, causing the R:FR ratio to drop to approximately 0.7-0.8 (Smith, 1982). This decrease of R:FR ratio can alter the phytochrome photoequilibrium, resulting in altered proportions of active or inactive phytochrome states.

The R:FR ratio exposure to a plant is also altered by the degree and proximity of competing vegetation. The largest change in spectral energy distribution of daylight is experienced under a canopy of vegetation. Light absorption, reflection and transmission of vegetation shows that most of the R and B region of the spectrum is absorbed by the carotenoids and chlorophylls (400-700 nm), whereas the FR region of the spectrum is either transmitted or reflected. Some green light is reflected or transmitted which makes chlorophyllous vegetation appear green to the human eye. Underneath the foliage the light that is transmitted through, or reflected from overhead vegetation, has a significantly reduced R:FR ratio (0.09-0.7) (Smith, 1982). A reduction in the R:FR ratio, caused by R being absorbed more strongly by vegetation than FR, provides cues to a plant, instructing it that other vegetation is in close proximity. This signal is transduced through a change in the relative proportions of Pr and Pfr isoforms, and acts as an unambiguous signal that competitors are nearby. The plant stem is the region where the majority of proximity detection occurs via the perception of horizontally reflected FR signals (Ballaré *et al.* 1990: Smith *et al*. 1990). When measuring irradiation under such a dense vegetational canopy, it is also noted that in addition to a change in R:FR ratio there is a decrease in photosynthetically active radiation (PAR). The photons traversing through the foliage and reaching the plant stem are depleted significantly at the level of the soil surface, and in shade-avoiding species such signals can result in the initiation of the appropriate modulations of growth and development in order to optimise survival.

1.3. R:FR ratio and Shade Avoidance

1.3.1. The shade avoidance syndrome

Light is a fundamental resource to plants as it is required for maintenance of a photosynthetically active state. Where plants are grown in close proximity to one another there will be constraints on the availability of light to drive photosynthesis. Light therefore becomes the limiting factor and plants compete for this key resource. The ability of an individual plant to find light dictates survival and thus reproductive potential. Plants have therefore evolved strategies, namely shade tolerance and shade avoidance, to promote survival in response to shaded environments. The majority of plants use shade avoidance strategies, exhibiting altered growth patterns in response to shade.

One of the first reports that phytochromes were required for shade avoidance came from Cumming in 1963 who suggested that R:FR ratio was important for optimising *Chenopodium rubrum* germination. Subsequently, the first suggestion that phytochromes mediate the shade avoidance response came from a series of experiments performed by Smith and colleagues in the 1970s. After measuring natural radiation spectra both above and below vegetation, the Smith group proposed that low R:FR ratio observed underneath the canopy would alter the photoequilibrium of phytochrome (Holmes and Smith 1975, 1977). This relationship was found to be hyperbolic, meaning that a reduction in R:FR ratio (i.e. under a vegetational canopy) will lead to a significant decrease in Pr. Subsequently, the role of R:FR ratio in the perception of vegetational shade was examined by growing plants in controlled artificial light environments. Such experiments were carried out using variable light quality conditions that resulted in unaltered photosynthetically active radiation (PAR).

By maintaining a consistent PAR, any observed changes in growth response could be attributed solely to altered R:FR ratio. It was important to maintain a consistent PAR as the as the metabolic resources utilized during shade avoidance depend upon the photosynthetic process. To maintain a constant PAR whilst varying R:FR ration, white fluorescent tubes were used in conjunction with supplementary FR light sources (Morgan and Smith, 1976, 1978, 1981). This model allowed a large number of physiological experiments to be carried out, thus leading to the discovery of a suite of developmental responses. It is also important to note that the results of these experiments mirrored observations made during vegetational shading studies in the natural environment (Morgan and Smith, 1979). It is also known that plants grown in an open habitat respond to a decrease in the R:FR ratio in a more pronounced way (Morgan and Smith, 1979). When analysing the responsiveness of plants to either natural vegetational shade or simulated shade conditions (WL plus supplementary FR), Smith and colleagues found a clear relationship between R:FR ratio, developmental response and phytochrome photoequilibrium. This suggested that the phytochromes are involved in physiological responses to changing R:FR conditions.

Morgan and Smith, (1979) documented a number of physiological responses following low R:FR ratio exposure. A clear morphological change in development is seen and the energy that is conserved within the plant for normal vegetative growth is reallocated and used for elongation growth. Elongation of the stem and petioles is observed, and this reorganisation of energy is thought to be at the expense of leaf and organ development. It has also been observed that when dicots are subjected to low R:FR ratio, an increase in apical dominance is seen which causes reduced branching (Casal *et al*, 1986). Another growth response to reduced R:FR ratio is upward

reorientation of leaves through hyponasty (Whitelam and Johnson, 1982). This response may be a predominant strategy to avoid shading by neighbours.

The kinetics of the stem elongation response was examined by Smith and colleagues using linear voltage displacement transducers which enabled real-time measurement of seedling elongation growth (Morgan *et al*, 1980). The group found that when WL grown *Sinapis alba* L was exposed to reduced R:FR light (through supplementary FR light), an increase in elongation growth would occur. Fibre optic light guides were then used to provide supplementary FR irradiation on specific *Sinapis alba* internodes, reducing the R:FR ratio on this area of the seedling alone. Similarly, this also caused rapid elongation which could be measured after 10-15 minutes. Interestingly, they found that if supplementary FR light was funnelled on just the leaves, elongation growth was still observed, but the kinetics of this response were much slower.

The stem elongation rate has since been found to be directly linked to the FR fluence rate. Morgan and colleagues 1980 showed that low R:FR ratio-induced growth could be reversed by increasing R light, thereby underscoring the potential importance of phytochrome photoequilibrium (Morgan *et al*, 1980). It was later found that supplementary FR given to *Sinapis alba* L at the end of day (EOD) only promotes elongation growth if given to leaves or cotyledons. Supplementary FR light given to the internode directly is not sufficient to cause elongation (Casal and Smith, 1988). Such an EOD-FR treatment has been clearly demonstrated to be a phytochrome dependent response. Elongation growth mediated by EOD-FR is primarily due to phyB. In wild-type plants the stable Pfr formed during the day continues to act through the night inhibiting hypocotyl growth; EOD FR removes most of this Pfr and subsequently hypocotyls elongate.

The concept that a reduction in the R:FR ratio incident upon an internode could cause a dramatic change in growth rate is in fitting with the notion of an early neighbour detection mechanism. Ballaré *et al*, (1987) discovered that plants could elongate their stems in response to a change in light quality before any reduction in photosynthetically active light could be detected. When *Chenopodium album*, *Datura ferox L.* and *Sinapis alba* seedlings were grown in full sunlight supplemented with a small amount of FR, available energy was redirected into stem elongation growth. Through phytochrome-mediated perception of R:FR ratio, plants were found to be able to detect the actual degree of shading and the potential threat of the surrounding plants competing for light. The ability of a plant to determine its relative spatial position within the community and mount an appropriate escape response if conditions are unfavourable is extremely important to its survival.

Subsequent experiments by Ballaré *et al* (1990) investigated FR signalling further by covering individual internodes of *Datura ferox* and *Sinapis alba* seedlings with cuvettes containing $CuSO_4$ thereby 'blinding' them to FR radiation. Blinded seedlings showed a severe attenuation in stem elongation responses, suggesting localised photomorphogenic control of stem elongation (Ballaré *et al*, 1990).

An acceleration in flowering is a hallmark response to reduced R:FR (Halliday *et al*, 1994). Unlike the immediate shade avoidance responses, flowering only occurs after prolonged exposure to low R:FR ratio light (see section 1.3.3. for more detail on regulation of flowering by R:FR ratio). Thus it can be envisaged that plants have discrete mechanisms for surviving both short and long term changes in R:FR. Short term changes induce the shade avoidance response which promotes growth and facilitates avoidance of canopy shade. Should this strategy fail, then the possibility of death (resulting from a long term change in R:FR) induces transition from vegetative

to reproductive development (Botto and Smith, 2002; Donohue *et al*, 2001; Dudley and Schmitt, 1995).

In agriculture, the shade avoidance syndrome is often linked to a reduction in productivity. Changes in R:FR ratio cause physiological adaptations which result in reallocation of resources from certain organs. A good model for this is the radish which, when grown in low R:FR ratio, displays a significant reduction in the bulb size. Arabidopsis and *Brassica*, when subjected to low R:FR ratio or EOD-FR treatments, show reductions in leaf area and whole plant biomass (Robson *et al*, 1993; Devlin *et al* 1996, 1999). Although shade avoidance is an important survival mechanism, it must be prevented in young seedlings until sufficient reserves are accumulated and it is thought that the phyA FR-HIR may provide such a mechanism (Smith *et al*, 1997; Yanovsky *et al*, 1998).

1.3.2. The role of different phytochromes in the shade avoidance response

As previously discussed, R:FR red ratio signals are perceived by phytochromes. Analysis of plants deficient in one or more phytochrome has enabled the deduction of their individual and over-lapping contribution to the shade avoidance response (see table 1.3.2.). The first two reported phytochrome-deficient mutants were the tomato *aurea* (*au*) mutant (Koornneef *et al*, 1985) and the cucumber *lh* (long hypocotyl) mutant (Adamse *et al*, 1987, 1988). However, subsequent analysis revealed that the *au* mutant was in fact a chromophore mutant. The characterisation of these mutants led to the speculation that different molecular species of phytochrome had different functions. Indeed, both species were found to respond differently to EOD-FR treatment: elongation growth in the *au* mutant (Koornneef *et al*, 1985) but not in the *lh* mutant. Further investigation by Lopez-Juez and colleagues (1988) demonstrated

that the *lh* mutant possessed wild-type levels of a light-labile phytochrome pool (possibly phyA). Immunohistochemical analysis revealed that *lh* mutants were deficient in a small phytochrome pool that remained stable in continuous light conditions. The *lh* seedlings also showed reduced cotyledon expansion and chlorophyll production; traits that are typical of wild-type seedlings grown in low R:FR ratio light. Genetic analysis revealed that the phenotype of *lh* mutants was due to lesion of a gene encoding a light stable phyB-like phytochrome (Adamse *et al*, 1988; López-Juez *et al*, 1992). Subsequent work has shown that other phyB-deficient mutants; for example the *Brassica rapa (ein) (elongated internode)* and the Arabidopsis *phyB* mutant; show a constitutive shade avoidance response in high R:FR ratio conditions. Taken together, these observations led to the conclusion that phyB-like proteins are required for shade avoidance signalling.

This initial demonstration that phyB-like deficient mutants show no response to EOD-FR or supplementary FR when compared to wild-type has since been discredited. It is now known that phyB-deficient mutants exhibit a detectable shade avoidance response under these lighting conditions. For instance, the hypocotyls of light-grown *lh* cucumber seedlings, although already elongated, show a small but significant, elongation response to supplementary FR light (Whitelam and Smith 1991; Smith *et al*, 1992). Such findings suggested that the *lh* mutation is either leaky, and therefore a residual but functional phyB-like product is expressed in these plants, or that additional phytochromes contribute to the R:FR ratio response. As mentioned previously, *ein* mutants deficient in phyB show an aberrant shade avoidance response however, when comparing internode elongation in *ein* mutants to wild-type, seedlings still display a reduced response to low R:FR ratio conditions. This clearly suggests that phyB is not the sole player in shade avoidance as although morphological

responses to light quality modifications are severely impaired in mutants deficient in phyB signalling, some physiological responses to light quality can still occur. Whilst it is now known that phyB is certainly not the only phytochrome capable of mediating shade avoidance, it is the principal mediator of elongation growth responses to high R:FR ratio conditions.

Arabidopsis phytochrome-mutant analysis has enabled a detailed description of the individual contribution of each phytochrome to R:FR ratio perception. The Arabidopsis Wassilewskija (Ws) ecotype harbours a homozygous 14-bp deletion in *PHYD* which codes for a truncated, non-functional phyD apoprotein. This strain has been used extensively to study the role of phyD in low R:FR ratio and EOD-FR responses. Adult monogenic *phyD* mutant responses to such treatments are comparable to those of wild type plants (Aukerman *et al*, 1997; Devlin *et al*, 1999). In contrast, double knockout of phyD and phyB has a marked effect on low R:FR ratio and EOD-FR responses: *phyBphyD* double mutants exhibit elongated petioles, reduction of cotyledon area, diminished response to the EOD-FR treatment and early flowering (Aukerman *et al*, 1997). Thus, phyD acts in concert with phyB to mediate shade avoidance. PhyD and phyB are a result of a relatively recent gene duplication event, so it is perhaps to not altogether surprising that they have some functional overlap (Goosey *et al*, 1997; Mathews and Sharrock, 1997; Devlin *et al*, 1999). They also share similar amino acid sequence and expression patterns, which provides additional evidence of a common function (Devlin *et al*, 1999). Devlin and colleagues (1999) found that phyD also acts in conjunction with phyC and /or phyE to control flowering time during prolonged shade avoidance.

Similarly, analysis of *phyA* mutants and *phyAphyB* double mutants has enabled conclusions to be made regarding functional redundancy of these two genes.

For example, in response to R light both the promotion of cotyledon expansion and induction of *CAB* (light harvesting Chlorophyll *a/b*) gene expression involves phyA and phyB. In the absence of one of these phytochromes these responses are little affected. However, these physiological responses are severely disrupted in *phyAphyB* double mutants. Double mutants have significantly elongated hypocotyls in low R:FR ratio conditions when compared to the monogenic *phyB*. This suggests that phyA inhibits hypocotyl elongation in the *phyB* mutant. In addition, *phyA* mutants show high seedling mortality under natural shade conditions/ low R:FR ratio conditions, an observation that suggests a role for phyA in controlling shade avoidance in small seedlings (Yanovsky *et al*, 1997). It is also known that phyA mutants flower late under long day conditions suggesting phyA involvement in the perception of day length (Johnson *et al*, 1994).

Notwithstanding these observations, the residual shade avoidance response; comprising acceleration of flowering and promotion of internode elongation between rosette leaves (Devlin *et al*, 1998); of *phyAphyB* double mutants lead to speculation that other phytochromes also contribute to this response. Subsequent genetic screens led to isolation of the *phyE* mutant. However, as the *phyE* monogenic mutant had no detectable phenotype in response to EOD-FR, *phyAphyBphyE* triple mutants were generated. When compared with WT, this mutant was constitutively early flowering and produced internodes between rosette leaves (Devlin *et al*, 1998). Phytochrome E is therefore implicated in responses such as flowering and internode length, the lack of phenotype in the monogenic mutant again suggesting genetic functional redundancy. In a *phyAphyB* background, *phyD* deficiency leads to elongation of petioles, whereas a *phyE* deficiency leads to internode elongation. This confirms that

phyE and phyD have discrete roles in light quality induced elongation growth responses.

The isolation and characterisation of *phyC*-deficient plants has shown that abrogation of phyC does not impair the shade avoidance response. Furthermore, *phyBphyC* double mutants also showed a shade avoidance phenotype that was comparable to *phyB* monogenic mutants. It is thought that phyC is involved in flowering and may be possibly involved in early seedling development (Franklin *et al*, 2003; Monte *et al*, 2003). At present it is clear that in Arabidopsis the perception of changes in R:FR ratio, and subsequent initiation of shade avoidance responses is mediated by phytochromes B, D and E.

Table 1.3.2. Summary of phytochrome functions.

Function	**Phytochromes**
Promotion of seed germination	phyA, phyB and phyE
Regulation of seedling de-etiolation	phyA, phyB, phyC, phyD and phyE
Suppression of root hair growth	phyB
Regulation of leaf architecture	phyA, phyB, phyC, phyD and phyE
Suppression of internode elongation	phyA, phyB and phyE
Antagonism of shade avoidance	phyA
Suppression of shade avoidance	phyB, phyD and phyE
Regulation of stomatal index	phyB
Repression of flowering	phyB, phyC, phyD and phyE
Entrainment of the circadian clock	phyA, phyB, phyD and phyE
Photoperiod perception	phyA and phyC

Table 1.3.2. Summary of phytochrome functions. (Adapted from Franklin and Quail, 2009).

1.3.3. Regulation of flowering by R:FR ratio

Floral induction is regulated by the phytochrome and cryptochrome photoreceptors. The transition to flowering in Arabidopsis is controlled by multiple regulatory pathways which converge to regulate the expression of floral meristem identity genes such as *LFY* (*LEAFY*) and *AP1* (*APETALA 1*) (Weigel *et al*, 1992; Bowman *et al*, 1993). The switch from vegetative to reproductive development must be precisely timed in order to maximise reproductive success (Simpson and Dean, 2002). A number of transcriptional regulators; such as *FLC* (*FLOWERING LOCUS C*) and *CO* (*CONSTANS*); integrate to control flowering genes such as *SOC1* (*SUPPRESSOR OF CONSTANS 1*) and *FT* (*FLOWERING TIME)* (Simpson and Dean, 2002).

An external coincidence model that receives convergent inputs from circadian and photoperiodic signals is responsible for triggering flowering specifically during long days. Daytime expression of CO is a central requirement for this response. Expression of this gene is induced by the circadian-regulated protein GI (GIGANTEA) and the circadian/ photoperiod regulated protein FKF1 ((FLAVIN-BINDING KELCH REPEAT F-BOX 1). However, *CO* expression is also strongly repressed by the circadian-regulated protein CDF1 (CYCLING DOF FACTOR 1). The ability of CDF1 to repress *CO* transcription can be overcome when GI and FKF1 associate to form a complex (Imaizumi *et al*, 2005). This physical interaction allows FKF1 to target CDF1 for degradation, thereby relieving repression of *CO* translation. Because this interaction depends upon activation of a blue-light sensing LOV domain on the FKF1 protein, CDF1 is only degraded when GI and FKF1 levels peak during daytime periods. In short days, *FKF1* transcript abundance peaks at night, and so *CO* transcription remains strongly repressed during daytime periods. However, in long days, *FKF1* levels peak during daytime. This results in GI-FKF1 complex formation

and degradation of CDF1, the net effect of which is an increase in CO abundance during daytime periods. Thus, it is only during long days, when periods of peak *FKF1*/*GI* expression coincide with daylight, that CO is able to induce *FT* and initiate floral inducing signalling cascades (Imaizumi *et al*, 2005; Samach *et al*, 2000). The process of flowering therefore requires the coincident expression of light dependent and circadian dependent genes.

When analysing *phyB* mutants it was discovered that the early flowering phenotype of these plants correlated with an increase of *LFY* and *FT* expression (Blάzquez and Wiegel, 1999; Cerdán and Chory, 2003: Halliday *et al*, 2003). Interestingly, it has since been shown that in low R:FR ratio conditions, the acceleration of flowering operates through *FT* independently of the transcriptional regulator *CO* (Halliday *et al*, 2003). The phytochromes are directly involved in controlling the floral promoter *FT* and have also shown that this early flowering phenotype is temperature dependent (Halliday *et al*, 2003). Mutagenesis screening has identified PFT1 (PHYTOCHROME AND FLOWERING TIME 1) as a potential signalling component linking phyB to *FT* expression (Cerdán and Chory, 2003). PFT1 displays a late flowering phenotype in both long and short day conditions and abnormal flowering responses to EOD FR light. The PFT1 protein, which localises in the nucleus, shows a degree of similarity to transcription factors. To further establish putative roles of PFT1 in the phyB/ FT signalling pathway, a *phyBpft1* double mutant was constructed (Cerdán and Chory, 2003). Whilst this double mutant had petiole lengths similar to *phyB* mutants, the plants showed no aberrant flowering response. Taken together, these findings suggest that there are many potential signalling mechanisms downstream of the phytochromes which operate together to regulate elongation growth and the onset of low R:FR ratio mediated early flowering. In

Arabidopsis flowering time is extremely flexible allowing the plant to adapt to its surrounding environment; it is adaptive to photoperiod and light quality, which altogether contributes to the plants reproductive success.

1.3.4. Interaction of phytochrome signalling with blue light sensing mechanisms

Blue light photoreceptors and R/FR phytochromes have been proposed to act synergistically to mediate physiological responses to light. Experiments show that B light responses, such as inhibition of hypocotyl elongation, can be partially reversed by pulses of saturating FR light (Casal, 1994). Such reversibility suggests that phytochrome and blue light signalling pathways converge. It has since been found that a threshold level of active phytochrome is necessary for cry1 to retain normal activity. This is demonstrated by the blunted blue light response of phytochrome mutants despite unaltered levels of CRY1 protein (Ahmad and Cashmore, 1997). Phytochrome-cryptochrome signalling pathways have been reported to be involved in the regulation of flowering time and input into the circadian clock (Mas *et al*, 2000; Devlin and Kay, 2000).

A number of shade avoidance-like responses are also observed in B light conditions, such as increased stem elongation in response to reduced quantities in B light (Ballaré *et al*, 1991 and Casal and Sánchez, 1994) and also the altering of tobacco leaf angle in response to B light (Pierik *et al*, 2004). The interaction between these two important photoreceptor systems is yet to be clearly defined. Interestingly, as demonstrated in chapter 5, the *XTH15* (*XYLOGLUCANENDOTRANS - GLUCOSYLASE /HYDROLASE*), a known R:FR ratio regulated gene, is also regulated by B light.

1.4. Signalling in shade avoidance

The integration of combined inputs to different photoreceptor classes converges to produce plant morphogenic changes. Although the photoreceptors involved in triggering shade avoidance behaviour have been well characterised, we know very little of the downstream targets that mediate this response. Physiological responses to low R:FR ratio can occur within a short time frame, an example of this is stem elongation of *S. alba* which occurs within 10 min of such light treatment (Morgan *et al*, 1980; Child and Smith, 1987). This rapid increase in stem elongation suggests that these responses must be mediated by either rapid changes in gene expression or by existing proteins within the plant. It is therefore important to know which genes are key downstream players in the shade avoidance response and how the temporal characteristics of their expression contributes to shade avoidance behaviour.

1.4.1. ATHB2

The first genes reported to be regulated by changes in R:FR ratio were the transcription factors *ATHB2* (also known as *HAT4*) and *ATHB4* (Schena and Davies, 1992; Steindler *et al*, 1999). Antisense *ATHB2* lines showed limited elongation in response to low R:FR ratio light whereas transgenic plants with elevated levels of *ATHB2* displayed some phenotypes similar to those of WT plants grown in reduced R:FR ratio (Steindler *et al*, 1999). These genes contain a homeodomain linked to a leucine zipper motif and have been shown to interact with the promoter element sequence CAATNATTG which suggests that they are transcriptional regulators (Sessa *et al*, 2005). It was found that in light-grown plants *ATHB2* transcript levels

were suppressed but in response to low R:FR ratio or EOD FR, de-repression of this gene occurred (Carabelli *et al*, 1993, 1996).

1.4.2. Identification of PIL1

In 2003, analysis conducted at the University of Leicester identified key molecular downstream targets of the phytochromes. Microarray analysis of 8,200 Arabidopsis genes was undertaken in plants exposed to a 1 h transient reduction in R:FR ratio at various time points (Salter *et al*, 2003). Hundreds of genes were found to show a change in gene expression in response to this light treatment. Amongst these, were *PIL1 (PHYTOCHROME INTERACTING FACTOR LIKE 1*) which showed a 35-fold increase in expression, *ENDO1* which showed an 8.4-fold increase in expression and a *DIN2* which showed a 16.1-fold increase in expression (Salter *et al*, 2003).

Both *PIL1* and *PIL2* (later identified as a homologue of *PIL1*) encode basic helix-loop-helix (bHLH) transcription factors. Genetic analysis revealed rapid increases in *PIL1* transcript after only 15 min of low R:FR ratio light whilst *PIL2* transcript abundance increased after 3 h of low R:FR ratio light. This suggests that *PIL2* it is not as rapidly regulated by changes in R:FR ratio as *PIL1* (Salter *et al*, 2003). PIL1 shows high homology to PIF3 (PHYTOCHROME INTERACTING FACTOR 3), a known DNA binding protein that binds directly to phyB. Despite this, PIL1 has been shown not to bind to PHYB in vitro (Khanna *et al*, 2004). Another interesting trait of both *PIL1* and *PIL2* is that de-repression of these genes by low R:FR ratio light is gated by the circadian clock, with maximum elevation of transcript occurring at around subjective dawn (Salter *et al*, 2003). This observation raises the interesting possibility that certain physiological responses to shade avoidance are gated by the circadian clock. A hypocotyl assay designed by Salter and colleagues

(2003) showed that a 2 h transient reduction of R:FR ratio causes a 30% increase in hypocotyl length, and that this response is controlled by the circadian oscillator. Hypocotyl elongation in day/ night cycles shows a maximum de-repression to low R:FR ratio at around subjective dawn and maximum repression at around subjective dusk (Lorrain *et al*, 2007). Mutants deficient in *PIL1* showed an aberrant hypocotyl elongation response to transient low R:FR ratio suggesting that *PIL1* is in part necessary for mediating this response. For more detail concerning the circadian clock's influence on shade avoidance genes, see section 1.5.

1.4.3. PIFS

A group of related bHLH-class transcription factors known as the PIFs (Phytochrome Interacting Factors) have been shown to physically interact with phyB in its light activated state. Additionally, some members of the PIF family also interact with phyA (Martinez-Garcia *et al*, 2000; Quail 2002; Nagatani, 2004 and Chen *et al*, 2005). Analysis of one of these transcription factors, PIF3, has demonstrated that it is critical for mediating phytochrome-induced regulation of gene transcription (Quail, 2002). PIFs were first suggested to interact with phytochromes in 1998 when Ni *et al* used the C terminal region of PHYB and PHYC as bait and PIF3 as prey in yeast two hybrid screens. This screen identified PIF3-phytochrome interactions which were subsequently confirmed by pull down assay. Although Pfr forms of both PHYA and PHYB bind to PIF3, they bind with markedly different affinities: PHYB has a ten-fold greater affinity than PHYA. Pull down assays were also used to determine the regions responsible for PHYB interaction with PIF3, revealing that deletion of 90 amino acids at the N terminus of PHYB virtually abolishes binding affinity for PIF3. Additional yeast two hybrid screens also demonstrated that in the presence of R light and

chromophore, the N terminal portion of PHYB can interact with PIF3 (Shimizu-Sato *et al*, 2002). It is now known that interaction between PHYB and PIF3 (and other PIF family members) is mediated by the active phytochrome binding (APB) domain. The APB domain is a small conserved N-terminal region that is crucial for mediating the interaction with phyB Pfr (Khanna *et al*, 2004).

Radiolabelling of PHYB has been used to investigate the temporal characteristic of PHYB-PIF3 interaction: a very brief (5 min) exposure to R light is sufficient to initiate PHYB-PIF3 interaction. In contrast, a sole FR light pulse or a R light pulse followed by FR light pulse does not cause PHYB-PIF3 association. This demonstrates that PHYB must exist in the Pfr form for the interaction to occur. It was also found that a R light pulse followed by dark incubation followed by a FR pulse causes dissociation of PHYB from PIF3. PIF3 localises in the nucleus irrespective of the light treatment (Ni *et al*, 1998). The bHLH domain of PIF3 is necessary for PIF3-PHYB interaction, as constructs in which this domain is deleted did not bind to PHYB (Zu *et al*, 2000).

In addition to physically interacting with one another, the phytochromes also appear to be able to regulate PIF3 protein levels. Phytochrome A, B and D act redundantly to reduce PIF3 levels. This effect can be attenuated in WT and completely reversed in the *phyA* mutants by a brief pulse of FR. This suggests that PIF3 is highly sensitive to degradation by Pfr as FR only causes a 0.1% of total PHYA to revert to this form (Al Sady *et al*, 2006). A separate study found that the degradation of PIF3 in response to FR light does not occur in *phyA* mutants but does in other phytochrome mutants (Bauer *et al*, 2004). This established that PHYA is responsible for FR light-induced PIF3 degradation. If seedlings are given FR light, transient formation of PIF3 nuclear speckles can be observed, but this diminishes after

ca. 6 hours. If seedlings are subsequently returned to darkness, speckles of PIF3 re-accumulate in the nucleus. Interestingly *cop1* mutant seedlings grown in the dark do not accumulate PIF3 to the same level as WT seedlings and what is present degrades rapidly in response to light. This indicated that COP1 is necessary for the proper accumulation of PIF3 protein levels within the nucleus but it is not necessary for its degradation (Bauer *et al*, 2004).

Phenotypically, PIF3 over-expressing lines display longer hypocotyls and smaller cotyledons under R light, whereas *pif3* mutants shows the opposite phenotype (Kim *et al*, 2003). Transgenic over expressing lines also show reduced cotelydon opening in both R and FR light. PIF3 has been suggested to be a positive or a negative regulator of phytochrome signalling depending on the light conditions (Kim *et al*, 2003).

Recent studies demonstrate that similarly to PIF3, both PIF4 and PIF5 interact preferentially with phyB. This was demonstrated by *in vitro* immunoprecipitation studies examining potential interactions between members of the PIF family and PHYB. In its Pfr form PHYB interacts with PIF4, PIF5 and PIF6 through the N-terminal APB binding motif. Mutation of any one of the four conserved residues in this domain has been show to abolish PIF5 protein association with PHYB. PIF1 (PHYTOCHROME INTERACTING FACTOR 1) is another known bHLH transcription factor that interacts with both phyB and phyA (Huq and Quail 2002; Huq *et al*, 2004; Khanna *et al*, 2004; Oh *et al*, 2004). PIF1 has the highest affinity for the phytochromes and is strongly sensitive to the quantity and quality of light. Phytochrome A is involved with the degradation of PIF1 following plants initial exposure to light and phyB and phyD are involved in PIF1 degradation after prolonged light treatments (Hui *et al*, 2008).

Both PIF4 and PIF5 have been found to be low R:FR ratio regulated. Recent work demonstrates that if WT seedlings are grown in short days and given low R:FR ratio at dawn or dusk, PIF5 protein expression is high. In contrast, if seedlings are exposed to high R:FR, PIF5 expression is significantly reduced. PIF4 protein has also been found to respond in a similar manner to PIF5 at dawn but not at dusk. This also coincides with maximum low R:FR ratio gene expression occurring around dawn (Lorrain *et al*, 2007).

Mutant and transgenic analysis has provided important clues regarding the role PIF5 plays during photoregulation of growth. *pif5* mutants are morphologically similar to WT when grown in WL light/ dark cycles. However, they do have shorter hypocotyls when grown in R light. In contrast, over expressing lines have phenotypes similar to *phyb* mutants: longer petioles, elongated primary inflorescences, large cauline leaves and early flowering in long and short days (Fujimori *et al*, 2004).

In WT seedlings, R light exposure decreases PIF5 protein expression. However, seedlings grown in the dark or in the presence of a proteasome inhibitor do not exhibit a decline in PIF5 levels (Lorrain *et al*, 2007). This suggests that phytochromes may be responsible for PIF5 degradation, a hypothesis supported by the observation that *phyAphyBphyD* triple mutants are unable to degrade PIF5. Phytochromes C and E are not thought to be involved in regulation of PIF5 turnover.

The *pif4* mutant has a short hypocotyl phenotype and over expression results in long hypocotyls in continuous R when compared to WT. The *pif4phyB* double mutant is similar to the *phyB* mutant indicating that PIF4, which localises in the nucleus, interacts with PHYB (Martinez Garcia *et al*, 2000). This has been validated by in vitro pull downs demonstrating PIF4–PHYB binding. PIF4 also shows a weak binding to PHYA (Huq and Quail, 2002). We now know that PIF4 and PIF5 are

targeted for degradation when bound to phytochrome. This degradation appears to be 36S proteosome-mediated, requiring the APB domain of the PIF4/PIF5 amino terminus. It is important to note that plants expressing PIF4 or PIF5 transgenes that have a deleted APB binding motif still show an elongated phenotype suggesting that these proteins are capable of positively regulating shade avoidance independently of phytochrome interaction (Lorrain *et al*, 2007).

The importance of PIF4 and PIF5 as regulators of elongation growth has been further confirmed by recent work showing that hypocotyl elongation is influenced by an external coincidence model, thus integrating PIF4 and PIF5 circadian regulation and light-mediated degradation (Nozue *et al*, 2007; Lorrain *et al*, 2007). In light/ dark grown plants transcript abundance of *PIF4* and *PIF5* is high from the end of the dark period through to the middle of the day but light induces rapid degradation of PIF4 and PIF5 proteins. Therefore maximum elongation growth is seen at the end of the dark period when the protein is most stable. This coincides with the time at which maximal responses to low R:FR ratio gene expression occur (Lorrain *et al*, 2007). Genetic studies therefore suggest that these bHLH transcription factors play both positive and negative roles in overlapping and distinct branches of light signalling pathways.

Table 1.4.3. Light specificity and biological functions of the PIF family members

PIF #	**Light Specificity**	**Phenotypes**	**Reference**
PIF1/PIF5	R/FR	Hypocotyl elongation Cotyledon expansion Hypocotyl gravitropism in dark Seed germination Chlorophyll biosynthesis	Huq, E. *et al*. (2004). Science 305, 1937–1941 Oh, E. *et al*. (2004). Plant Cell 16, 3045–3058
PIF3	R	Hypocotyl elongation Cotyledon expansion Hypocotyl gravitropism in dark Anthocyanin biosynthesis Chlorophyll biosynthesis	Ni, M. *et al*. (1998). Cell 95, 657–6676 Oh, E. *et al*. (2004). Plant Cell 16, 3045–3058 Monte, E. *et al*. (2004). Proc. Natl. Acad. Sci. U. S. A. 101, 16091–16098 Kim, J. *et al*. (2003). Plant Cell 15, 2399–2407
PIF4	R	Hypocotyl elongation Cotyledon expansion	Huq, E. and Quail, P.H. (2002). EMBO J. 21, 2441–2450
PIF5/PIL6	R	Hypocotyl elongation Cotyledon expansion	Khanna, R. *et al*. (2004). Plant Cell 16, 3033–3044 Yamashino, T. *et al*. (2003). Plant Cell Physiol. 44, 619–629 Fujimori, T. *et al*. (2004). Plant Cell Physiol. 45, 1078–1086
PIF6/PIL2	Unknown	Unknown	Khanna, R. *et al*. (2004). Plant Cell 16, 3033–3044 Yamashino, T. *et al*. (2003). Plant Cell Physiol. 44, 619–629

1.4.4. HFR1

HFR1 (*LONG HYPOCOTYL IN FR 1*) encodes a bHLH transcription factor that was initially identified as a positive regulator of phyA signalling (Fairchild *et al*, 2000). Through analysis of double mutants between *hfr1* and different blue light photoreceptor mutants, it was demonstrated that *HFR1* is a component of the CRY1 blue light signalling pathway (Duek and Fankhauser, 2003). Microarray analyses have shown that low R:FR ratio provokes a rapid induction of *HFR1* (Sessa *et al*, 2005). Recent studies have indicated that in *hfr1* null mutants, R:FR ratio regulated genes show a greater level of transcript in response to prolonged low R:FR ratio treatments. This is accompanied by an aberrant response to the phyA-mediated FR light response (Fairchild *et al*, 2000; Fankhauser and Chory, 2000). Taken together these data strongly suggest that HFR1 acts as a negative feedback mechanism for the shade avoidance response. Thus HFR1 may act to ensure that physiological responses to shade are not exaggerated (Sessa *et al*, 2005). In contrast, it has also been suggested that, given the antagonistic role of phyA in shade avoidance (Salter *et al*, 2003), the *hfr1* mutant phenotype may simply reflect a defect in normal phyA signalling (Franklin and Whitelam, 2006).

1.4.5. Other genes identified to be regulated by a change in R:FR ratio

Devlin *et al*, 2003 carried out microarray analysis on global changes in gene expression in wild-type, *phyB* and *phyA* seedlings after either 1 h or 24 h of low R:FR ratio treatment. 92 genes of wild-type seedlings exposed to low R:FR ratio light showed changes in relative expression that were greater than 2-fold. Interestingly, among these were a number of auxin-related genes, which show antagonistic regulation by phyA and phyB in response to a change in light quality. Two of these

genes were members of the auxin transport encoding PIN family proteins (Friml *et al*, 2002) PIN3 and PIN7. A number of auxin-induced transcription factors *IAA1*, *IAA3*, *IAA5*, *IAA11* and *IAA19* are also regulated in a similar fashion. It is now known that light regulates a complex transcriptional cascade which starts with the perception of a change in R:FR ratio. This signal is then transduced by the phytochromes, resulting in regulation of gene expression by a number of different transcription factors.

1.5. Circadian rhythms

Circadian rhythms are a subset of biological rhythms with a period, defined as the time to complete one 24 h cycle. The first documentation of a circadian rhythm in plants came from Androsthenes in the fourth century BC. He observed that the leaves of *Tamarindus indicus*, exhibited a horizontal position during the day and a more vertical position at night. However, it was not until 1729, when the French astronomer Jean Jacques d'Ortous de Mairan reported that *Mimosa pudica* leaves could follow a similar pattern of movement in constant darkness, was it postulated that an endogenous clock controls leaf movement behaviour (de Mairan, 1979).

A defining attribute of circadian rhythms is that they are endogenous and self-sustaining. This means that they can persist under constant environmental conditions (continuous light, dark, temperature) (Millar and Kay 1991). It is only in laboratory conditions where an organism can be subjected to artificial environmental time cues, such as day/night cycles or temperature changes. Environmental cues are termed zeitgebers (time givers). Zeitgebers normally entrain the circadian clock to a period of approximately 24 h. This period corresponds to the cycle of the Earth's rotation. Under continuous darkness, temperature or light, a free running period is still

observed. It is important to note however, that certain stimuli reset the endogenous clock in order to allow for synchronisation with day/ night cycles (phase), so that the organism can anticipate dawn and dusk. In plants, components of the photosynthetic machinery accumulate each day in anticipation of dawn. Before dusk, leaves and flowers often close up in order to protect delicate tissue from the potentially damaging effects of lower temperatures that occur at night (Darwin, [1895]1981; Enright, 1982).

For most organisms the period of the circadian clock is not quite 24 h as its timing is adjusted slightly with each dawn and dusk event. This plasticity allows an organism to continually adapt to its surrounding environment. An example of this is the ability to adjust to changing day length as the seasons change throughout the year. The clock reacts differently to light at different times during a day; a pulse of light given before dawn will advance the phase of the clock, yet the same pulse of light given after dusk will delay the phase. If the same pulse of light is given during subjective day, it will have no effect at all. This is essential as although the clock is sensitive to light (in order to permit resetting), it must not be sensitive to very short pulses of light (such as a flash of lightening occurring at night) as this would cause aberrant resetting of the clock. Therefore in general, a relatively prolonged pulse of irradiation is required to reset the clock (Nelson and Takahashi, 1991). It is also true if photon irradiance increases, the free running period of the circadian rhythm decreases. Therefore the brighter the light the faster the circadian clock runs. For nocturnal organisms the opposite is true (Aschoff, 1979).

The circadian clock is often thought of as having three major components, an input pathway, whereby the environment synchronises the clock, an endogenous oscillator and an output pathway whereby rhythmic metabolic systems controlled by the clock are coordinated. This is however a simplistic model, as none of these

components operate in isolation: the output pathway for example modulates (gates) the input pathway (Devlin, 2002). A large range of physiological responses are controlled by the circadian clock including cotyledon and leaf movement (Engelmann *et al*, 1994; Millar *et al*, 1995) photoperiodic induction of flowering (Devlin and Kay, 2000), stomatal opening (Somers *et al*, 1998) and hypocotyl elongation (Millar *et al*, 2003).

To date, the majority of plant circadian rhythm studies have utilized the clock controlled gene *CAB* as a marker of circadian cycling. The CAB protein forms part of the photosynthetic machinery of the plant and, as would be predicted for a clock controlled gene, accumulates prior to dawn, showing a peak of expression early in the day and a decline towards dusk (Millar and Kay, 1991). In 1992 the *CAB* promoter was fused with the firefly luciferase reporter gene (*LUC*). This *CAB::LUC* construct was transformed into plants (Millar e*t al*, 1992) and a sensitive photon counting camera was used to monitor rhythmic expression of the protein. This paradigm led to isolation of several Arabidopsis clock mutants (Millar e*t al*, 1995). The circadian clock was found to consist of three interlocked feedback loops with two single myb domain transcription factors. The genes *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*) and *LHY* (*LATE ELONGATED HYPOCOTYL*) both play a role in each loop. TOC1 (TIMING Of CAB 1) the founding member of a family of *PRRs* (*PSEUDO-RESPONSE REGULATORS)* closes one loop while three *TOC1* paralogs *PRRS, PRR7* and *PRR9* close a second loop. A third loop includes a myb transcription factor *GI* (*GIGANTEA*) (Locke *et al*, 2006; Mas and Yanovsky *et al*, 2009) (Figure 1.5).

toc1 loss of function mutants were identified as a short clock period mutants. This led to the discovery that oscillating mRNA and protein levels of clock components such as TOC1 are necessary for the central oscillator to function

correctly. This is supported by the observation that *TOC1* over-expressers are arrhythmic. The gene *CCA1* is also essential for normal clock function (Green and Tobin, 1998; Wang and Tobin, 1999). Loss of *cca1* function, like that of *LHY*, confers a short period whilst the *cca1lhy* double mutant is arrhythmic, suggesting these clock components function redundantly (Alabadí *et al*, 2002; Mizoguchi *et al*, 2002). How these genes form an oscillator loop is not completely understood. CCA1 and LHY negatively regulate *TOC1* through *TOC1* promoter region binding and act as negative regulators of *TOC1*TOC1 is thought to be a positive regulator, since expression of *CCA1* and *LHY* is greatly reduced in a *toc1-2* mutant (Alabadí *et al*, 2002; Mizoguchi *et al*, 2002, Harmer and Kay, 2005; Hazen *et al*, 2005). TOC1 contains a CCT (CONSTANS, CONSTANS LIKE, TOC1) domain thought to be involved in transcriptional regulation (Strayer *et al*, 2000) but it has not been shown to bind either CCA1 or LHY promoters. Several other genes including *GI*, *ELF3* (*EARLY FLOWERING 3*), *ELF4* (*EARLY FLOWERING 4*) and *LUX*, have also been found to be required for *CCA1* and *LHY* expression (Park *et al*, 1999; Doyle *et al*, 2002; Mizoguchi *et al*, 2002; Hazen *et al*, 2005).

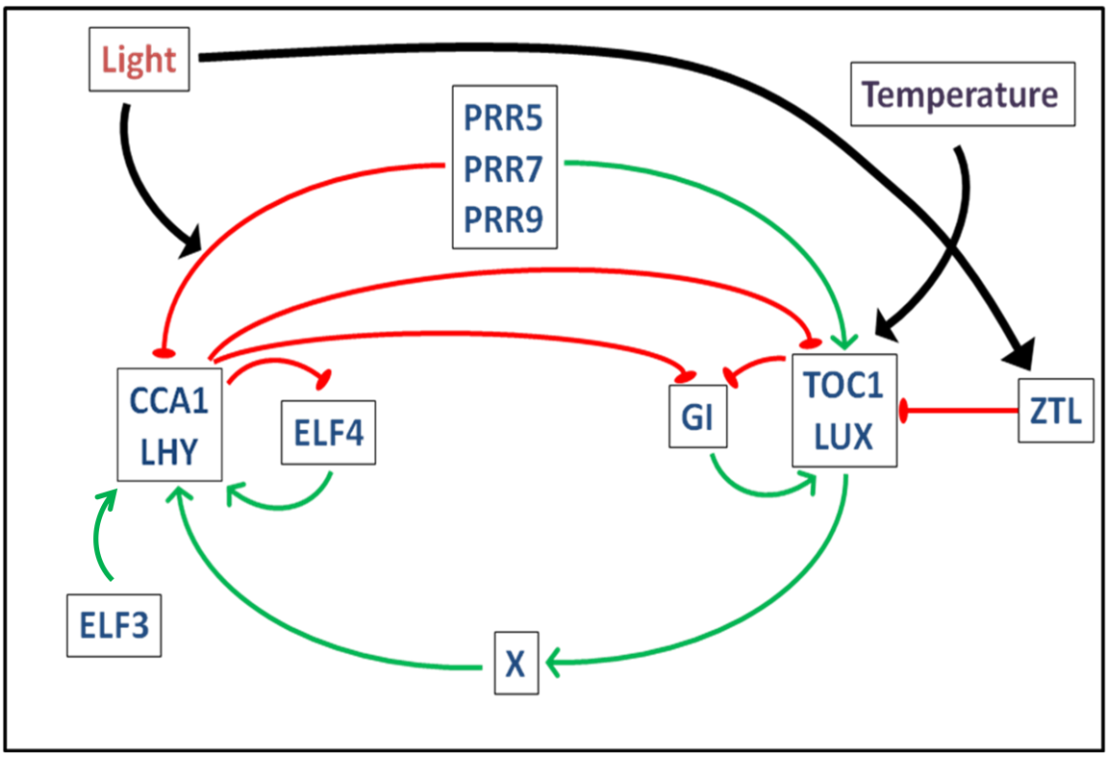


Diagram 1.5. A simplified model of the core oscillator adapted from Hotta *et al*, 2007. Arrows indicate a positive relationship between components and bars indicate a negative relationship. Component X is predicted from Mathematical modelling. CCA1 (CIRCADAIN CLOCK-ASSOCIATED 1), ELF3 (EARLY-FLOERING 3), ELF4 (EARLY-FLOWERING 4), GI (GIGATEA), LHY (LATE ELONGATED HYPOCOTYL), LUX (LUX ARRHYTHMO), PRR5 (PSEUDO-RESPONSE REGULATOR 5), PRR7 (PSEUDO-RESPONSE REGULATOR 7), PRR9 (PSEUDO-RESPONSE REGULATOR 9), TOC1 (TIMING OF CHLOROPHYL A/B BINDING PROTEIN 1), ZTL (ZEITLUPE).

The photoreceptors that comprise the input pathway are involved in resetting of the circadian clock. Somers *et al*, (1998) demonstrated that both phytochrome and cryptochrome contribute to light input to the clock in Arabidopsis through crossing *CAB::LUC* transgenic lines with null mutants for *PHYA*, *PHYB*, *CRY1* and *CRY2*. Phytochrome B was found to be important at high fluences of R light, whereas phyA was found to function as a low fluence photoreceptor (Somers *et al*, 1998). This spectrum of detection by phyA and phyB demonstrates the plasticity of the photoreceptors when detecting a range of fluence rates. Cryptochrome 1 functions in high fluence blue light and both phyA and cry1 function at low fluence rates.

Although *cry2* monogenic mutants show wild-type circadian responses to blue light input (Somers *et al*, 1998), the *cry1cry2* double mutant shows redundancy between cry1 and cry2 at intermediate fluence rates (Devlin and Kay, 2000). This suggests redundancy between cry1 and cry2 at intermediate fluence rates (Devlin and Kay, 2000). This redundancy is also reflected in seedling development, as both cry1 and cry2 act together in lower fluence conditions. In contrast, at higher fluence rates cry2 is degraded leaving cry1 as the blue light photoreceptor involved in de-etiolation (Lin *et al*, 1998). Interestingly the *cry1* mutant shows deficiency in the perception of low fluence R light. The fact that cryptochromes show no absorption peak in the R light region of the spectrum suggests that *CRY1* is acting as a downstream signal transduction component of PHYA (Devlin and Kay, 2000).

Evidence for the action of *PHYD* and *PHYE* in R light input to the clock has also been demonstrated. These proteins show conditional redundancy with *PHYB:* Their involvement only becomes apparent in the absence of phyB (Devlin and Kay, 2000). The photoreceptor mutants have no effect on the circadian clock in darkness, clarifying that these genes only play a role in the light input pathway. Other

candidates involved in the light input pathway downstream of the photoreceptors have recently been identified: ZTL (ZEITLUPE) forms part of a small family of three closely related proteins along with FKF1 (FLAVIN-BINDING, KELCH REPEAT, F-BOX 1) and LKP2 (LOV KELCH PROTEIN 2) (Nelson *et al*, 2000; Kiyosue and Wada, 2000). Whilst ZTL mRNA transcription is not clock regulated, protein expression is; peaking at around dusk and troughing at around dawn. ZTL is an F box protein that poly ubiquitinates specific targets, thereby facilitating their degradation. ZTL is a component of the SCF complex, which recruits TOC1 for proteasomal degradation. In plants lacking functional ZTL, levels of TOC1 are elevated and no longer subject to circadian-dependent degradation. In contrast, over-expressing ZTL decreases period length. Therefore *TOC1* abundance is controlled at the transcriptional level by LHY and CCA1 and the protein level by ZTL.

Another candidate signal transduction component downstream of phytochrome in light input to the clock is PIF3. RNA blot analysis of PIF3-deficient seedlings indicates that PIF3 is necessary for phyB induced expression of the photoresponsive genes *CCA1* and *LHY*. Both the *CCA1* and *LHY* promoter regions contain PIF3-binding G-box motifs. As PIF3 also binds phyB, regulation of *CCA1* and *LHY* is likely to require the presence of a phyB- PIF3 complex. It has therefore been proposed that PIF3 might represent the central control point through which the phytochromes regulate photomorphogenesis and circadian clock entrainment (Martinez-Garcia *et al*, 2000).

Transcription of Arabidopsis *PHYB* and *CRY1/ CRY2* genes oscillates with a circadian rhythm in continuous light (Bognar *et al*, 1999; Harmer *et al*, 2000). PhyB shows a peak around subjective dawn, whilst the cryptochromes show a peak at a later stage in the subjective day (Harmer *et al*, 2000). This suggests that these

photoreceptors contribute to both input and output pathways of the circadian clock. Such modulation, or 'gating' of light responsiveness over the course of a circadian cycle is not only observed for the resetting of the clock, but also by the output pathway which can be directly regulated by light (Millar and Kay, 1996).

1.5.1. The circadian gating of R:FR ratio responses

R:FR ratio responses have also been found to be gated by the circadian clock (Salter *et al*, 2003). The circadian clock is known to regulate hypocotyl elongation: When seedlings are grown in continuous light, hypocotyls show maximum elongation at subjective dusk. In contrast, when plants are grown in light dark cycles, hypocotyls show maximum elongation at dawn. It has now been found that many genes regulated by altered R:FR ratio are also regulated by the circadian clock. When seedlings are given a R:FR ratio treatment, regardless of the time of day, genes such as *PIL1* are de-repressed and an increase in transcript is observed. However, a strong circadian rhythm in light responsiveness of *PIL1* de-repression is observed, thus indicating that light signalling to *PIL1* (or other R:FR ratio regulated genes) is strongly gated by the circadian clock (Salter *et al*, 2003).

Indeed, it is now clear that many genes regulated by a reduction in R:FR ratio are in fact also regulated by the circadian clock. The pioneering studies within this field suggested that many of the R:FR ratio, circadian regulated genes show a peak in transcript at dawn, when in fact it was thought that physiological responses were not seen until dusk (Salter *et al*, 2003). The environmental importance of the dusk elongation process has now been challenged: Recent work strongly suggests that elongation observed at this time was an artefact of exposing seedlings to continuous light conditions throughout the course of the experiment. When seedlings are grown

in light dark cycles similar to those observed in the environment, maximum elongation growth does not occur at dusk, but rather early in the morning, thereby coinciding with the time at which maximum light-regulated gene expression is observed (Lorrain *et al*, 2007).

1.6 Background to the project

The aim of this study is to further our understanding of the shade avoidance signalling pathway. The focus of this thesis is on a number of genes (*DIN2*, *ENDO1* and *XTH15*) whose expression has previously been shown to be regulated by R:FR ratio. The impetus to study *DIN2* and *ENDO1* arose from previous microarray analysis implicating these genes as components of the shade avoidance signalling cascade (Salter *et al*, 2003). This study analysed R:FR ratio dependent changes in expression of 8200 *Arabidopsis* genes. Whilst hundreds of genes were found to show altered expression, the most significantly affected were *DIN2* and *END01*. Furthermore, a literature based investigation into the potential roles of these genes suggested that both *DIN2* and *ENDO1* could have roles in cell wall modification, a known response necessary in shade avoidance. The third gene chosen for study, *XTH15*, is a member of a family of genes thought to play a role in the physiological cell wall modifications. *XTH15* was initially investigated by the Whitelam group at the University of Leicester as the lab wished to gain insight into downstream targets involved in the shade avoidance phenotypic response. This preliminary work demonstrated that *XTH15* transcript was strongly regulated by low R:FR ratio. Thus, it was postulated that this gene could be involved in the cell wall modifications that occur during elongation growth under low R:FR ratio light conditions. To conclude, whilst preliminary

evidence suggested these genes were regulated by changes in R:FR light, their role in shade avoidance had not been investigated in detail.

1.6.1. Aims

This study comprises three chapters, which examine the potential roles of *DIN2*, *ENDO1* and *XTH15* in shade avoidance. My aim was to elucidate the molecular mechanisms by which these genes are regulated and how they are involved in shade avoidance behaviour. Determination of the photoreceptors that regulate their expression was through analysis of various phytochrome and cryptochrome mutants. Possible downstream transcriptional regulators of these genes were subsequently determined by examining the influence of several transcription factors known to play a key role in the shade avoidance signalling pathway. In addition, the possibility that expression of *DIN2*, *ENDO1* and *XTH15* is modulated by the circadian clock was explored through molecular analysis. Finally, the possible role that *DIN2*, *ENDO1* and *XTH15* play in shade avoidance was addressed using mutant analysis and over expressing lines, with an aim to determine the connection between the light mediated regulation of these genes and potential biological function.

In the three results chapters that follow, data is presented which was obtained from experiments conducted between October 2003 and December 2006. Upon completion of my studies, nothing was known of the role these genes in shade avoidance signalling. In the time that has since lapsed, the field has progressed and many exciting advances have been made (See Chapter 6 for an overview of these recent advances). The work described in this thesis together with these recent advances are

facilitating a comprehensive understanding of the molecular machinery underpinning shade avoidance regulation.

2. Materials and Methods

2.1. Plant growth

Arabidopsis thaliana cv. Columbia or *Arabidopsis thaliana* cv. Landsberg *erecta* and *Nicotiana tabacum* cv. Samsun were used for all experiments unless otherwise stated.

2.1.1. Plant growth media.

Lehle medium, for *in vitro* growth of Arabidopsis seedlings, was prepared by mixing 5 ml 1 M KNO_3, 2.5 ml 1 M KH_2PO_4, 2 ml 1 M $MgSO_4$, 2.5 ml Sequestrene (2.5 g $FeSO_4.7H_2O$ and 3.3 g Na_2EDTA in 400 ml sdH_2O which was brought to the boil and allowed to cool on a magnetic stirrer for ~30 min before adjusting the final volume to 450 ml), 1 ml micronutrients (70 mM H_3BO_3, 14 mM $MnCl_2$, 0.5 mM $CuSO_4$, 1 mM $ZnSO_4$, 0.2 mM $NaMoO_4$, 0.1 mM $CaCl_2$) in a final volume of 1 l. Bio Agar was added at 0.6% before autoclaving and the medium was stored at room temperature. Appropriate antibiotics were added at the following concentrations: kanamycin 40 µg ml^{-1}, Basta 10 µg ml^{-1}. For sucrose-containing media 3% (w/v) sucrose was added to the Lehle medium. The medium was then autoclaved and stored at room temperature.

2.1.2. In vitro culture

Seeds were sown on Lehle medium (Section 2.1.1.) and then stratified for five days at 4°C in the dark before being transferred to a growth room and grown at 22°C under 12 h light/ 12 h dark at a photon irradiance of approximately 100 µmol m^{-2} s^{-1}. If adult Arabidopsis wild-type and mutant plants were required for experimental analysis or adult plants were required to set seed, seedlings were transferred to 24 compartment trays 7-10 days after germination and kept covered with a propagator lid for 4-6 days following transfer. Each pot contained compost: sand at a ratio of 3:1. Plants were

grown under 12 h light/ 12 h dark cycles at 22°C (unless otherwise stated) in a controlled temperature room. Controlled long day and short day experiments were carried out in the Vindon cabinets (Vindon Scientific Ltd, IIII HS), where both temperature and day-length can be accurately regulated. The irradiance was typically 100 μmol m^{-2} s^{-1}.

Seeds collected from Arabidopsis plants that had been transformed (section 2.4.3.) and Arabidopsis seeds which were grown on 3% sucrose-containing Lehle medium, were surface-sterilised by first shaking in a 10% bleach (Super bleach steriliser, Coventry Chemicals Ltd) solution for ~20 min followed by washing six times in sdH_2O. Sterilised seeds were dried on filter paper in a laminar flow hood and sown on the appropriate Lehle medium (Section 2.1.1.).

2.1.3. Soil culture

Seeds germinated on soil were grown in 5 cm Petri-dishes without lids inside a moist chamber. They were grown in Levingtons F2 Seed and Modular Compost and silver sand. Each Petri dish contained compost: sand at a ratio of 3:1. Seeds were stratified for 5 days at 4°C in the dark. Seeds for experiments where synchronous germination was required were exposed to a 1h white light (120-150 μmol m^{-2} $s^{-1)}$ pulse to induce the germination of the seeds; this was followed by the return of the plates to darkness for a further 24h at 22°C. Plates were then subjected to the appropriate experimental conditions. All plants grown in 5-cm Petri plates were watered from the top using water bottles. All adult plants were watered from the base.

For Arabidopsis plants grown for *Agrobacterium* transformation (Section 2.6.3.), seeds were sown directly onto soil. Pots were covered with aluminium foil and placed at 4°C for 2-4 days before being transferred to the greenhouse. The aluminium

foil covering the pots was then removed and replaced with a standard propagator lid until the seedlings were fully germinated. Arabidopsis seeds were harvested by breaking dry siliques over a piece of paper and then sifting the seed through mesh to remove dry plant material.

Tobacco seeds were germinated on soil in the same way as for Arabidopsis seeds but were grown in a greenhouse at under long-day conditions and the photon irradiance was typically 150-200 μmol m^{-2} s^{-1}.

2.1.4. Plant transformation

Transgenic Arabidopsis were generated using *Agrobacterium*-mediated transformation protocol based on the floral dip method (Clough and Bent, 1998). Pots which were 81 cm^2 were sown with 10 plants per pot and grown in a greenhouse. The primary inflorescences were normally cut when they reached 5 cm to promote the generation of secondary inflorescences. The plants were used for transformation when the secondary inflorescences reached no more than 10 cm in height and had a few open flowers.

See section 2.4.3. for the method for the transformation of the constructs into Arabidopsis using *Agrobacterium tumefaciens* strain GV3101. For each construct a starter culture was initiated by inoculating 5 ml of LB supplemented with 50 μg ml^{-1} gentamycin, rifampicin 50 μg ml^{-1} and 50 μg ml^{-1} kanamycin with one fresh *Agrobacterium* colony, this starter culture was incubated at 28ºC for 24 h in a shaking incubator. A total of 4 ml of the starting culture was used to inoculate 1 litre of LB split between 2 conical flasks containing the same antibiotics as the starting culture. Cultures were again incubated at 28ºC in a shaking incubator and grown until the absorbance was A_{600} = 1.8-2.0 (~16 h after inoculation). The cultures were centrifuged

at 4000 rpm for 15 min in a GSA rotor (Sorvall R-C6) at room temperature. The pellet was resuspended in 1 litre of dipping medium (0.5 % w/v sucrose and 10 mM $MgCl_2$) and 0.05 % Silwet L-77 added immediately prior to dipping. The suspended culture was split between 500 ml shallow jars and the Arabidopsis inflorescences immersed for 15 min by dipping the Arabidopsis plants upside down and submerging them in the jars. The excess liquid was gently shaken from the plants and the plants were laid down on their side and loosely covered with cling film to provide a humid environment. After 24 h the film was removed and the plants were grown under standard conditions until the siliques were dry and the seed ready for harvesting. The seed was bulk harvested and the first generation was screened for transformants. Screening for T_1 seeds was performed on Lehle medium containing 40 μg ml^{-1} kanamycin. Kanamycin-resistant seedlings were selected 10 days after germination and transferred to fresh Lehle plates before being transplanted to soil.

2.2. Light sources

2.2.1. R:FR ratio experiments

Low R:FR ratio experiments were performed using arrays of far red LEDs (λmax 735 nm Shinkoh electronics). Plants maintained in high R:FR ratio received a photon irradiance of 400-700 nm at approximately 100 μmol m^{-2} s^{-1} and a R:FR ratio of 4.7. The low R:FR treatments received the same photon irradiance, but a R:FR ratio of 0.089 (R:FR ratio range 0.03).

2.2.2. Hypocotyl assays in high R:FR ratio and low R:FR ratio

Seeds were germinated on soil (2.1.3.) in 5 cm Petri-dishes without lids placed inside a moist chamber. Seedlings were grown in high R:FR ratio 12 h light/ 12 h dark for 5 days. On day number 6 seedlings were given a transient low R:FR ratio pulse of 2 h commencing 2.5 h before dusk. The seedlings were then returned to high R:FR for 30 min. Controls remained in high R:FR. After 24 h, seedlings were removed and placed on agarose plates and measured (2.2.7.).

2.2.3. EOD FR treatments

Seeds were germinated on soil (2.1.3.) in 5 cm Petri-dishes without lids placed inside a moist chamber. Seedlings were grown in high R:FR ratio 12 h light/ 12 h for 5 days. On day number 6 seedlings were given a transient 20 min FR pulse (photon irradiance 10 μmol m^{-2} $sec^{-1)}$ commencing 3 min before lights off. This transient EOD FR treatment occurred for three days. Seedlings were then measured see section 2.2.7.

2.2.4. Petiole, leaf area and circadian experiments on adult plants

Seeds were germinated on soil (2.1.3.) in 5 cm Petri-dishes without lids inside a moist chamber. Seedlings were grown in high R:FR ratio 12 h light/ 12 h for 7 days. Seedlings were transferred to 24 compartment trays 7 days after germination and kept covered with a propagator lid for 4-6 days following transfer. After 14 days they were then moved to either high or low R:FR ratio 12 h light 12 h dark for a further 7 days. The plants were then measured (2.2.7.). For the circadian experiment seedlings were grown for 21 days and on day 22 they were moved to continuous high R:FR ratio and the relevant treatment was given at various time points.

2.2.5. Monochromatic light sources

Far red light was achieved by placing plates in a box containing FR light emitting diodes (LEDs) at a λmax of 735 nm (Quantum Devises, Barneveld, WI, USA). Red light was achieved by placing plates under R LEDs at a λmax of 665nm (Farnell, Leeds, UK). Blue light was obtained by placing plates in a blue light rig constructed with Blue LEDs at a λmax of 460nm. Measurements of irradiance were made using a hand held LI-COR Quantum sensor, model LI-250 for measurement of R and B light, and a portable EPP2000 Stellarnet spectroradiometer for measurement of FR and R:FR ratios. Seedlings were measured according to section 2.2.7.

2.2.6. Photon irradiance experiments under monochromatic light

Seeds were germinated on Lehle medium contained in 5 cm Petri-dishes. The Petri-dishes were then transferred into black plastic boxes with clear lids. The position of these boxes within the cabinets and the number of layers of Whatmann 3mm paper in the lid of the boxes was adjusted to provide the required photon irradiance.

2.2.7. Physiological measurements of Arabidopsis

Seedlings were laid out on agarose plates and photographed using a Nikon Digital camera D50. Hypocotyl length, petiole length, cotyledon and leaf area were measured using image processing and analysis software, ImageJ 1.32j. Flowering time was recorded as the number of rosette leaves present when plants displayed a 1-cm inflorescence stem.

2.3. Mutant analysis

The experiments carried out with the *pif* and pil mutants used the following protocol. Seeds were germinated on soil (2.1.3.) in 5 cm Petri-dishes without lids placed inside a moist chamber. Seedlings were grown in high R:FR ratio 12 h light/ 12 h dark for 5 days. On day number 6 seedlings were given a transient low R:FR ratio pulse of 1 h commencing at dawn.

The *pif3, pif4* and *pif5* mutants and PIF3, PIF4 and PIF5 over expressers were obtained from Professor C Fankhauser (University of Lausanne). The *pil1* mutant, PIL1 over expresser and phytochome mutants were obtained from Professor G Whitelam (University of Leicester).

2.4. *GUS* histochemical analysis

2.4.1. XTH15::GUS construct

An *XTH15::GUS* transgenic line was donated by J, Becnel, M, Natarajan, A, Kipp and J, Braam in Rice University, Houston, Texas. The seedlings were selected using the kanamycin resistance marker gene. Seedlings were selected on Lehle medium containing 40 μg ml^{-1} kanamycin.

2.4.2. Histochemical localisation of GUS activity

Seedlings were grown under 12 h light/ 12 h dark cycles at 22°C in both high R:FR and low R:FR for 5 days. The seedlings were then harvested and immediately submerged into a GUS staining buffer (Caissard *et al*., 1994) with 1mg/ml 5-bromo-4-chloro-3-indoyl-ß-D-glucuronic acid (X-gluc) (Melford Laboratories LTD, Ipswich, UK) and placed under a vacuum for 15 min. The seedlings were then incubated for a

further 24 h in darkness at 37 °C. Chlorophyll was removed by submerging the tissue in 50% ethanol for 1-2 h. The 50% ethanol was then replaced with 100% ethanol and the seedlings were incubated overnight in darkness. After 24 h, the ethanol was replaced with 50% ethanol for a further 1-2 h. Finally, all ethanol was removed and 1-2 ml of 50% glycerol was added (Jefferson *et al*, 1987). The seedlings were then mounted onto glass slides using 80% glycerol. Staining was visualised using a Leica LED2000 microscope. Colour images were captured on a Nikon D100 digital camera and processed in Adobe Photoshop version 7.0.

2.5. Bacterial work

2.5.1. Growth and storage of bacteria

The bacterial strains used in this study are listed in Table 2.5.1. *Escherichia coli* (*E. coli*) were cultured in liquid LB media (10 g NaCl, 10 g Tryptone and 5 g Yeast extract were combined in a final volume to 1 l, autoclaved and stored at room temperature) in a shaking incubator at 250 rpm, or on LB-agar plates (1.5 % agar was added to LB media before autoclaving). *Escherichia coli* cultures were grown at 37 ºC. *Agrobacterium tumefaciens* (*A. tumefaciens*) strain GV3101 was also grown in LB or on LB-agar but in incubators set at 28°C. When appropriate, antibiotics were added at the following concentrations: 50 μg ml^{-1} ampicilin; 50 μg ml^{-1} kanamycin; 50 μg ml^{-1} spectinomycin. For blue-white selection 5-bromo-4-chloro-3-indolyl -D-galactoside (X-gal) was added to the LB-agar plates at a final concentration of 0.5 mg ml^{-1}.

For short term storage (up to 3 months) cells were streaked onto LB-agar plates and stored at 4°C. For long term storage bacterial glycerol stocks were produced by

mixing 500 µl of an overnight culture with 500 µl of sterile 50% glycerol in a microcentrifuge tube. The glycerol stock was snap frozen in liquid nitrogen and stored at -80°C for up to three years.

Table 2.5.1. Bacterial strains used in this study

	Genotype	**Source/reference**
E. coli DH5α	*endA1 hsdR17* ($r_k^- m_k^-$), *supE44, thi1, recA1, gyrA(Nal^r), relA1*, Δ*(lacZYA-argF)*U169, Φ*80lacZ*Δ*M15*	Novagen
A. tumefaciens GV3101	Contains the disarmed pTiC58 plasmid pMP90RK	Monsanto

2.5.2. Escherichia coli transformation

Transformation-competent *E. coli* DH5α were made using the following procedure: DH5α from glycerol were streaked onto a fresh LB-agar plate. A single colony from the plate was used to inoculate 5 ml of LB medium and was incubated at 37ºC overnight in a shaking incubator. In total 1 ml of the saturated overnight culture was used to inoculate 100 ml LB medium in a 250 ml conical flask, this culture was incubated until optical density (OD) was A_{600} = 0.5 (2-3 h). The culture was transferred to chilled centrifuge tubes and centrifuged for 5 min at 3600 rpm at 4°C in a SS-34 rotor (Sorvall R-C6). After centrifugation the supernatant was discarded and the pellet resuspended in 40 ml of chilled $MgCl_2$ (0.1 M). The culture was centrifuged for 10 min at 3600 rpm at 4ºC and the pellet resuspended in 40 ml chilled $CaCl_2$ (0.1 M). The tubes were then stored on ice for at least 30 min. The culture was again centrifuged for 10 min at 3600 rpm at 4 ºC and the supernatant was discarded. The pellet was then re-suspended in 4 ml MOPS glycerol (100 mM MOPS-NaOH, 50 mM

$CaCl_2$, 20 % w/v glycerol; autoclaved and stored at 4°C). The chemically competent cells were dispensed into 100 µl aliquots in 1.5 ml centrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C.

To transform *E. coli*, aliquots of chemically competent cells in 1.5 ml centrifuge tubes were thawed on ice for ~5 min. 5 µl of plasmid DNA was added to the cells and mixed by gentle pipetting. The cells were incubated on ice for ~15 min before being subjected to heat shock at 42ºC for 90 sec in a water bath followed by incubation on ice for 3 min. A volume of 200 µl of LB medium was added to each transformation and the cells are allowed to recover at 37ºC with shaking. A total of 100 µl of each transformation was spread onto an LB-agar plate containing appropriate antibiotics and X-gal and incubated at 37°C overnight.

2.5.3. Transformation of Agrobacterium tumefaciens

Competent *Agrobacterium* was prepared using the freeze-thaw method (An *et al*, 1988). A starter culture was initiated by inoculating 5 ml of LB supplemented with 50 µg ml^{-1} gentamycin and rifampicin µg ml^{-1} with one fresh *Agrobacterium* colony. The following day 2 ml of the starter culture was used to inoculate 50 ml LB in a 250 ml conical flask and grown to an A_{600} of 0.5-1.0. The cells were then chilled on ice for 10 min before harvesting at 3,000 rpm for 5 min at 4°C in a SS-34 rotor (Sorvall R-C6). The supernatant was discarded and the cells gently resuspended in 1 ml chilled 20 mM $CaCl_2$ solution. The cells were dispensed into 100 µl aliquots in 1.5 ml pre-chilled centrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C.

For each transformation an aliquot of competent cells was put on ice and 1 µg of plasmid DNA immediately added. The cells were then thawed by incubating the tube in a 37°C water bath for 5 min, mixing gently half way through. Subsequently 1

ml of LB media was added and the cells incubated at 28°C with gentle shaking (100 rpm) for ~4 h. A volume of 150 µl of the cells were then spread on an LB-agar plate containing the appropriate antibiotic and incubated at 28°C for 2-3 days.

2.6. Molecular biology

All DNA was stored at -20°C and RNA was stored at -80°C. Unless otherwise stated all room temperature centrifugation steps were carried out in an Eppendorf Centrifuge 5415 D, using a F45-24-11 fixed angle rotor, at maximum speed (16,110 x g; 13,200 rpm). All centrifugation-steps were carried out at 4°C in an Eppendorf Centrifuge 5417, using f-45-30-11 fixed angle rotor, maximum speed (25,000 x g; 16,400 rpm). Large volumes of bacterial cultures were centrifuged in a Sorvall refrigerated RC-6 centrifuge.

2.6.1. Nucleic acid preparation

2.6.1.1. Plant DNA extraction

The method used for DNA extraction from Arabidopsis is adapted from Edwards *et al*., (1991). One leaf per plant was harvested and snap frozen in liquid nitrogen. Extraction buffer (500µl) was then added (200 mM Tris (pH 7.5), 250 mM NaCl, 25 mM EDTA (pH 8.0), 0.5 % (w/v) SDS) and the leaf is ground using a pellet pestle, (Sigma), samples were vortexed briefly to dispense large clumps. Samples were centrifuged for 5 min at 4ºC. A volume of 400 µl of supernatant was mixed with 400 µl of isopropanol. Samples were incubated at -80ºC for 30 min followed by centrifugation for 6 min at 4ºC. The supernatant was removed and the pellet was

rinsed with 70% ethanol and allowed to dry at room temperature for 20 min. The pellets were resuspended in 50 µl of sdH_2O. The samples were then centrifuged briefly and the supernatant removed to a fresh 1.5 ml centrifuge tube, 1-2 µl of sample was routinely used in PCR.

2.6.1.2. Plant RNA extraction

Plant RNA extractions were carried out using the Nucleo spin RNA II ABgene, Total RNA Miniprep Kit in accordance with the manufacturer's instructions. RNA extracted through this method was treated with Deoxyribonuclease I (DNase I) to remove any contaminating DNA during this protocol.

2.6.1.3. Plasmid preparation from bacterial overnights

Plasmid DNA was prepared from *E. coli* cultures using the GeneElute^{TM} Plasmid Miniprep Kit (Sigma) or the QIAprep Spin Miniprep kit (Qiagen) in accordance with the manufacturer's instructions.

2.6.1.4. DNA extraction from agarose gels

DNA fragments were resolved by agarose gel electrophoresis (Section 2.3.5.1.) and visualised on a Benchtop trans-illuminator (Syngene). The desired band was excised from the gel using a sharp razor blade and excess agarose removed. The DNA was extracted from the gel slice by using the QIAquick Gel Extraction Kit (Qiagen) in accordance with the manufacturer's instructions.

2.6.1.5. Quantification of DNA and RNA

The concentration of double-stranded DNA (dsDNA) and RNA solutions was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. An aliquot of the stock samples was diluted 1:200 in sdH_2O and the spectrophotometer standardised using sdH_2O in a quartz cuvette. Measurements were made for each sample at A_{260} and A_{280}. The ratio A_{260}/A_{280} was used to estimate the purity of nucleic acid, since proteins absorb at 280 nm. A ratio between 1.8-2.0 meant that the level of contaminants was at an acceptable level. Concentrations of dsDNA and RNA were calculated using the equations:

1 OD unit A_{260} nm = 50 µg/ml dsDNA

1 OD unit A_{260} nm = 40 µg/ml RNA.

2.6.2. Enzymatic manipulation of nucleic acids

2.6.2.1. Endonuclease restriction of nucleic acids

For restriction enzyme digests, between 0.5-1 µg plasmid DNA or 200 ng PCR product was used. Restriction enzymes and buffers were obtained from New England Biolabs and the recommended buffer used at 1x concentration in the reaction. At least 1 unit of restriction enzyme was used per microgram of DNA. Reactions were typically 10 µl total volume and digests were incubated at the appropriate temperature for ≥ one hour. To stop the reaction the digests were incubated at 65°C or 80°C for 20 min, as per the manufacturer's instructions. After digestion a small quantity of the reaction (typically 2 µl) was analysed on an agarose gel and the remaining DNA was ethanol precipitated and then the subsequent treatment performed.

2.6.2.2. Dephosphorylation of 5' ends

Shrimp Alkaline Phosphatase (SAP) (Roche) was used to catalyse the dephosphorylation of 5' phosphates from linearised plasmid DNA. Approximately 50 ng of vector DNA was included in a reaction with 0.9 µl of 10x Dephosphorylation Buffer (supplied by Roche) and 1U SAP and the reaction mixture made up a final volume of 9 µl with sdH_2O. The reactions were incubated at 37ºC for 10 min followed by inactivation of SAP through incubation at 65ºC for 15 min and the linearised plasmid DNA used directly in ligations.

2.6.2.3. DNA ligations

Ligations were performed using T4 DNA ligase (Invitrogen). T4 DNA ligase catalyses the joining of two strands of DNA between the 5' phosphate and the 3' hydroxyl groups of adjacent nucleotides. Polymerase Chain Reaction products were ligated into pDrive cloning vector (Qiagen) using the cloning kit ligation protocol. Blunt ended PCR products were purified using the StrataPrep® PCR Purification Kit (Stratagene) or SureClean solution (Bioline) as per the manufacturer's instructions. For ligations into the pDrive Cloning vector the following reaction was prepared: 1 µl pDrive cloning vector, 5 µl of ligation master mix, 2x, 4 µl of PCR product, the reaction volume was made up to a final volume of 10 µl with sdH_2O. The insert : vector ratio was typically 50:1. The ligation reactions were incubated at 16ºC for ~1 hour. All other ligations were carried out by combining 50 ng of linearised, dephosphorylated vector, 4 µl of 5x ligase buffer (supplied by Invitrogen), 0.5 µl T4 DNA ligase (5 U. μl^{-1}) and 2–6 µl of insert DNA fragment in a final volume of 20 µl. The insert: vector ratio was typically 3:1 and all ligations were incubated at 16°C over night followed by transformation into chemically competent *E. coli* (Section 2.4.2).

2.6.2.4. Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was used to look for difference in transcript of certain genes in different light conditions, also to screen bacterial colonies for the presence of plasmids, screen Arabidopsis insertion line. For all general analytical PCR, *Taq* PCR Master Mix Kit (Qiagen) was used. For all applications a reaction volume of 20 µl was used. The reaction mixture consisted of: 10 µl *Taq* PCR Master Mix, 0.5 µl of each primer (50 µl stock) and 1 µl of template DNA. The reaction was made up to 20 µl with sdH_2O.

For colony PCR the reaction volume was made up to 20 µl with sdH_2O and a small amount of bacterial colony added to the PCR reaction by touching a toothpick against a colony and then dipping the toothpick in the PCR tube. For cloning applications Accuzyme (Bioline) a high fidelity proof reading polymerase was used following the manufactures instructions. A total of 1 ng plasmid DNA or 1 µl first strand cDNA (Section 2.3.2.5.) was used as a template in these reactions.

The thermal cycling program used for PCR amplification was preceeded by as denaturing step of 95ºC for 5 min followed by 35 cycles (analytical) or 20 cycles (cDNA amplification for cloning) of: (i) denaturation for 30 sec at 95°C; ii) primer annealing for 30 sec between 50-65°C; iii) elongation at 72°C for 0.5-2 min depending on the size of the expected product (60 sec per kb). The cycling program was followed by 72°C for 5 min to promote completion of partial extension products and annealing of single-stranded complementary products and the PCR reaction was held at 4ºC until analysis.

2.6.2.5. RT-PCR

RT-PCR was used to clone cDNAs and also to analyse transcript levels. All RNA was quantified following the procedure in section 2.3.1.5. First strand cDNA synthesis was carried out using Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) (Promega). For each sample 2 µg of RNA was used. 2 µg RNA and 2 µl oligo dT primer (0.5 µg μl^{-1}) in a total volume of 15 µl sdH_2O was incubated at 70ºC for 5 min to melt secondary structures within the template. Samples were cooled immediately on ice. To the annealed primer template solution the following components were added in the order shown: 5 µl M-MLV 5x Reaction buffer (supplied by Promega), 1.25 µl dNTPs (10 mM each) 1 µl M-MLV RT (200 U) and sdH_2O to a final volume of 25 µl. The reaction mixture was incubated at 42 ºC for 60 min before the enzyme was inactivated at 70°C for 15 min. A volume of 1 µl of first-strand cDNA synthesis reaction was used as a template in each 20 µl PCR reaction.

2.6.2.6. Quantitative RT-PCR

For each sample a 20 µl reaction was set up: 10 µl Sigma SYBR® Green JumpStart™ Taq ReadyMix™, 1 µl of each 5 mM primers, 2 µl of first strand cDNA, made up to the final volume with sdH_2O. Reactions were set up using a robot (Corbett Research CAS1200) and Quantitative RT-PCR was performed using a MJ Research Chromo 4™ QPCR machine using the program: Incubation at 94ºC for 2 min followed by a cycle of 94ºC for 30 sec, 63ºC for 30 sec, 72ºC for 30 sec for 40 cycles. At the end of each cycle the plate is read measuring an increase in fluorescence. After the completion of the cycling program a melting curve from 75ºC to 90ºC with a read every 0.2ºC is performed to check for the production of more than one PCR product. Data was analysed using Opticon Monitor software.

Relative quantification was determined according to the equation (Pfaffl, 2001):

$$\text{Ratio} = \frac{(\text{Etarget})^{\Delta CP \text{ target (control-sample)}}}{(\text{Eref})^{\Delta CP \text{ target (control-sample)}}}$$

where E= PCR efficiency = $10^{(-1/\text{slope})}$, target = target gene, reference $^{(ref)}$ = *ACTIN*, control = wild-type, and sample = target sample. All data is represented as mean ± sem of 3 technical repeats.

2.6.2.7. DNA sequencing

All DNA sequencing was carried out by John Innes Centre Genome Laboratory (John Innes Centre, Norwich). A volume of 20 µl of plasmid (100-200 ng/µl) in sdH_2O was sent for sequencing. The sequencing results were viewed using EditSeq (DNA Star) and Chromas (Technelysium) and assembled using SeqMan (DNA Star).

2.7. Mutant Identification and generation of over-expressing lines

2.7.1. Obtaining homozygous lines

Knockout mutants for *DIN2*, *ENDO1* and *XTH15* unless otherwise stated were obtained from the Nottingham Arabidopsis Stock Centre. *din2-1* = SALK_029737, *din2-2* SALK_105913, *endo-1* = SALK_043675, *xth15-1* = SALK_039468 and *xth15-2* = SALK_099819. All SALK lines contain a T-DNA insert in the open reading frame. Homozygous lines were generated using the recommended PCR test from SIGnAL T-DNA verification primer design website (http://signal.salk.edu/tdnaprimers.html). By carrying out RT-PCR using three primers (LBb1, LP and RP) for SALK line testing, wild-type seedlings have product size of approximately 900-110. For homozygous lines a band will be seen which has a product size of approximately 410 bps plus N (N = the difference of the actual

insertion site and the flanking sequence position and for heterozygous lines both bands are seen.

A second mutant knockout line for *endo1-2* was obtained from the Institut National De La Recherche Agronomique. The Flag line (FST number 380H07) also contained a T-DNA insertion in the open reading frame. Seedlings contained a kanamycin resistance marker and were therefore grown on kanamycin 40 μg ml^{-1} and primers designed to verify the T-DNA insert. A homozygous line was then obtained.

2.7.2. Generation of over-expressing lines

Dark induced 2 over-expressing lines (DIN2 OX) and *XTH15* (XTH15 OX) over-expressing lines were constructed in the Col ecotype by sub-cloning the *DIN2* and *XTH15* cDNA into the plant transformation vector pROK2 under the control of the CaMV 35S promoter. The cDNA was amplified using *Taq* PCR Master Mix Kit (Qiagen) (2.6.2.4.). PCR products were ligated into pDrive cloning vector (Qiagen) (2.6.2.3.). DNA sequencing was then carried out by John Innes Centre Genome Laboratory (2.6.2.7.) The gene was then cut out (2.6.2.1.) of pDrive and ligations (2.6.2.3.) were carried out in order to insert the gene of interest into pROK2. The Plant transformation was carried out (2.5.3.) seed were then collected and transformed lines (T1) were then selected using the resistance marker Kanamycin 40 μg ml^{-1}. A resistant seedling (T2) was obtained and was grown up for seed collection. Seeds were then grown on plates containing Kanamycin 40 μg ml^{-1}. Segregation of seedlings was observed. A resistant seedling was then grown and seed was collected (T3). The seed collected was then found to show 100% resistance on Kanamycin 40 μg.ml^{-1}. Quantitative RT-PCR was then carried out in order to analyse transcript in the DIN2 OX and XTH15 OX relevant to wild-type.

2.8. Localisation Studies

2.8.1. Particle bombardment of tobacco

Particle bombardment (biolistic transformation) was used for the direct gene transfer into the leaf tissue to transiently express YFP fusion proteins in tobacco leaves. This technique involves accelerating DNA-coated gold particles (the microcarriers) directly into intact tissues. To prepare the micro carriers for particle bombardment 60 mg of 1.0 μm gold microcarriers (Bio-Rad Laboratories) were suspended in 1 ml of 70% ethanol by vortexing for 5 min and then left to settle for 15 min. The gold particles were harvested by pulse centrifugation for 5 sec, the ethanol was removed and the particles washed 3 times with sdH_2O. The gold particles were again harvested and resuspended in 1 ml of 50% glycerol and stored at -20 ºC.

To coat the DNA onto the gold micro carriers the glycerol stock was vortexed for 5 min and 20 μl of gold microcarriers were transferred to a clean tube. While vortexing 12 μl of plasmid DNA (10 μg/μl), 10 μl of 5 M CaCl2 and 8 μl of 0.1M spermidine (free-base) were added to the gold followed by vortexing for 2 min. The mixture was incubated on ice for 30 min and then the microcarriers were pelleted by pulse centrifugation for 2 sec at maximum speed. The supernatant was carefully removed without disturbing the pellet and the pellet washed by adding 100 μl 70% ethanol and inverting the tube gently ten times. This wash step was repeated with 100 μl ethanol. The DNA coated micro carriers were thoroughly resuspended in 20 μl of ethanol by pipetting and 6 μl was spread over the centre of the macrocarrier (BIO-RAD Laboratories). Particle bombardment was carried out using a PDS-1000/He^{TM} Helum biolistic particle delivery system (Bio-Rad Laboratories), which is powered by a burst of helium gas to accelerate the microcarriers into the sample. All

transformations were performed using 1100 psi rupture discs (Bio-Rad Laboratories) under 25 in Hg vacuum. Tobacco leaves were placed on Lehle medium. For each transformation the sample was bombarded with microcarriers twice and stored in the dark at room temperature for 24-48 h before image acquisition.

2.9. Microscopy

2.9.1. Fluorescence microscopy

Plant samples expressing YFP fusion proteins were generated using particle bombardment (Section 2.8.1.). All plant samples were then prepared for analysis in the same way: a small amount of tobacco leaf tissue (~1 cm^2) was placed on a microscope slide with sdH_20, covered with a 20x40 mm coverslip and firm pressure applied to remove air bubbles.

2.9.2. Image acquisition

Samples were viewed using Nomarski Differential Interference Contrast (DIC) microscopy. Colour images were captured on a Nikon D100 digital camera and processed in Adobe Photoshop version 7.0. Fluorescence image acquisition was performed on a Nikon TE-2000U inverted fluorescence microscope equipped with an Exfo X-cite 120 fluorescence illumination system (Exfo) and filters for YFP (exciter HQ500/20, emitter S535/30), emitter HQ680/40) (Chroma Technologies, USA). Images were captured using a Hamamatsu Orca ER 1394 cooled CCD camera. Standard image acquisition and analysis was performed using Openlab (Improvision).

3. Endochitinase Like 1

3.1. Introduction to the chitinases

Sequencing and systematic automated annotation of the Arabidopsis genome has led to the discovery of 24 putative chitinase-encoding sequences. According to the glycosyl hydrolase classification system, which is based on amino acid sequence similarity in catalytic domains, these chitinases belong to families 18 and 19 (Henrissat, 1991). Family 18 chitinases are found in bacteria, fungi, yeast, viruses, plants and animals, whereas family 19 members are almost exclusively present in plants. Interestingly, chitinases of both families do not share sequence similarity and so are likely to have arisen from a different ancestor (Hamel *et al*, 1997). Family 19 chitinases hydrolyse GlcNAc-GlcNAc and GlcN-GlcNAc linkages (Ohno *et al*, 1996). Plant chitinases are divided into six different classes based on sequence similarity to their tobacco orthologs (Meins *et al*, 1994). Chitinases from classes' I, II and IV belong to family 19.

Chitin is the main component of the insect exoskeleton, of crustacean shells and of the cell wall of many fungi but is absent in plants. Thus, traditionally, it was thought that plant chitinases act as a defence against pathogens that contain chitin in their cell wall. Supporting evidence for this hypothesis comes from transgenic studies which demonstrate that increased expression of chitinases reduces susceptibility to specific pathogens (Broglie *et al*, 1991; Jach *et al*, 1995).

Notwithstanding these studies, recent work shows that the role of chitinases is not restricted to pathogen defence as their expression is also induced by stress and plant hormones (Navabpour *et al*, 2003; Teruaki *et al*, 2004). Interestingly, it is also thought that chitinases are likely to play a role in the physiological development of plants: several studies show that chitinase expression is developmentally-regulated,

with specific isoforms being expressed in discrete organs during finite stages of development. For example, Arabidopsis *AtEP3/AtchitIV* is thought to be involved in embryogenesis and pollen development (Passarinho *et al*, 2000). At later stages of development, *AtEP3* is distributed in other regions of the plant indicating it has differential roles in embryonic and postembryonic plants (Passarinho *et al*, 2000). At least one chitinase-like protein, ELP1 (EDM2 LIKE PROTEIN 1), is believed to be essential for normal Arabidopsis development. ELP1 loss-of-function mutants have ectopic lignin deposition, aberrant cell shape with incomplete cell walls, reduced hypocotyl length, increased hypocotyl width, and root hairs that are increased in number and length (Zhong *et al*, 2002). The *ELP1* gene is expressed in all organs during normal plant growth and development (Zhong *et al*, 2002)

Chitinases show homology to 'yieldin', a protein that has been implicated in cow pea plant cell wall modifications (Okamoto-Nakazawa *et al*, 2000a; Okamoto-Nakazawa *et al*, 2000b). Yieldins are wall-bound proteins that are thought to have novel regulatory roles in cell wall yielding. They regulate the driving force of wall yielding, thereby exerting a direct influence on growth rate (Okamoto-Nakazawa *et al*, 2000a). It is therefore plausible that certain chitinases are involved in regulating cell wall development.

3.1.1. Endochitinase Like 1 a class IV Arabidopsis chitinase

ENDO1 ((ENDOCHITINASE 1) At2g43570) is a putative chitinase, belonging to glycosyl hydrolase family 19 with the structural characteristics of a class IV Arabidopsis chitinase. In the Arabidopsis genome, there are nine sequences that show class IV chitinase characteristics (figure 3.1.1.). Four of these encode pseudogenes whilst the other five, including ENDO1, are likely to be secreted active chitinases. To

date At3g54420, encoding *AtEP3/AtchitIV*, is the only class IV chitinase in Arabidopsis that has been studied in detail and shown to play a role in plant development (Passarinho *et al*, 2000). Gerhardt *et al*, 1997 proposed that class IV chitinases are likely to play a role in plant defence against pathogens. However, evidence for this hypothesis came from studying plant species other than Arabidopsis. It has since been concluded that class IV chitinases may have multiple functions, but in Arabidopsis it seems these proteins are more likely to be involved in developmental processes rather than in defence reactions (Passarinho *et al*, 2002).

This chapter focuses on the regulation of *ENDO1*, a glycosyl hydrolase family 19 member with the structural characteristics of a functionally active class IV chitinase, by R:FR ratio. In particular, it seeks to determine which photoreceptors are involved in *ENDO1* regulation, the role of the circadian oscillator in controlling abundance of its transcript, and finally the possible biological role of this gene in the shade avoidance response.

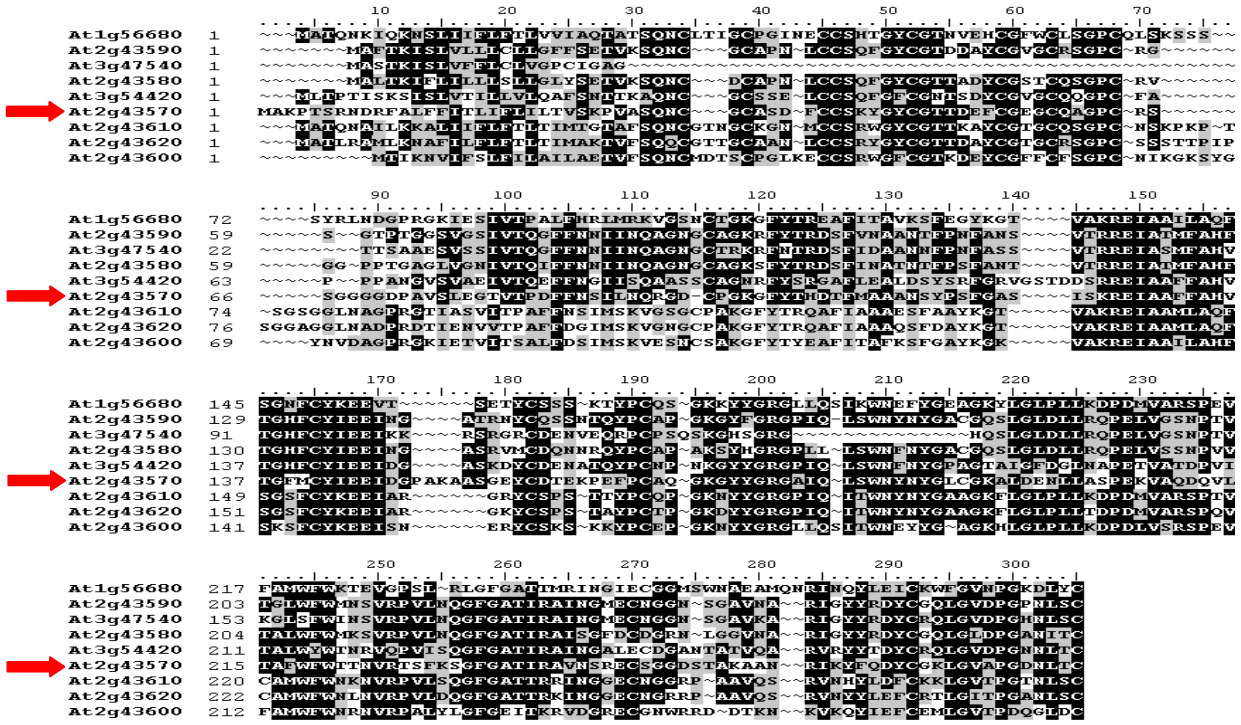


Figure 3.1.1. Multiple sequence alignment of Arabidopsis class IV chitinases. Gaps were introduced in order to allow optimal alignment and the degree of shading represents the level of similarity. The alignment was constructed using BioEdit Sequence Alignment Editor Software. The figure was then processed using Adobe Photoshop version 7.0. The red arrow indicates ENDO1.

3.2. Results

3.2.1. R:FR ratio regulation of ENDO1

ENDO1 was initially found to respond to a change in R:FR ratio following Affymetrix oligoarray analysis (Salter *et. al*., 2003 (for further detail see section 1.4.1.)). Relative expression of *ENDO1* increased 8.4 fold in response to a 1 h low R:FR ratio treatment administered 1 h after dawn. This finding suggested that *ENDO1* may be involved in the rapid shade avoidance response.

In order to confirm these results, experiments were repeated using Columbia seedlings grown on soil plates described in section 2.1.3. Seedlings were grown in 12 h light/ 12 h dark high R:FR ratio conditions for 5 days. At dawn one plate of seedlings were moved into low R:FR ratio conditions for 1 h, whilst the controls remained in high R:FR ratio. A further subset of seedlings were then given 1 h low R:FR ratio treatment 1.5 h prior to dusk. Tissue was then harvested and Quantitative RT-PCR conducted (Section 2.6.1.2., 2.6.2.6.). The results confirm that *ENDO1* is regulated strongly by a reduction in R:FR ratio (Figure 3.2.1.). Furthermore, expression of *ENDO1* was found to be differentially regulated at dawn and dusk. Relative expression of *ENDO1* in response to 1 h low R:FR ratio pulse at dawn resulted in a 16 fold change whereas the same 1 h low R:FR ratio treatment at dusk caused an 8 fold change in transcript to occur. This finding will be discussed in relation to the circadian clock in section 3.5.1.

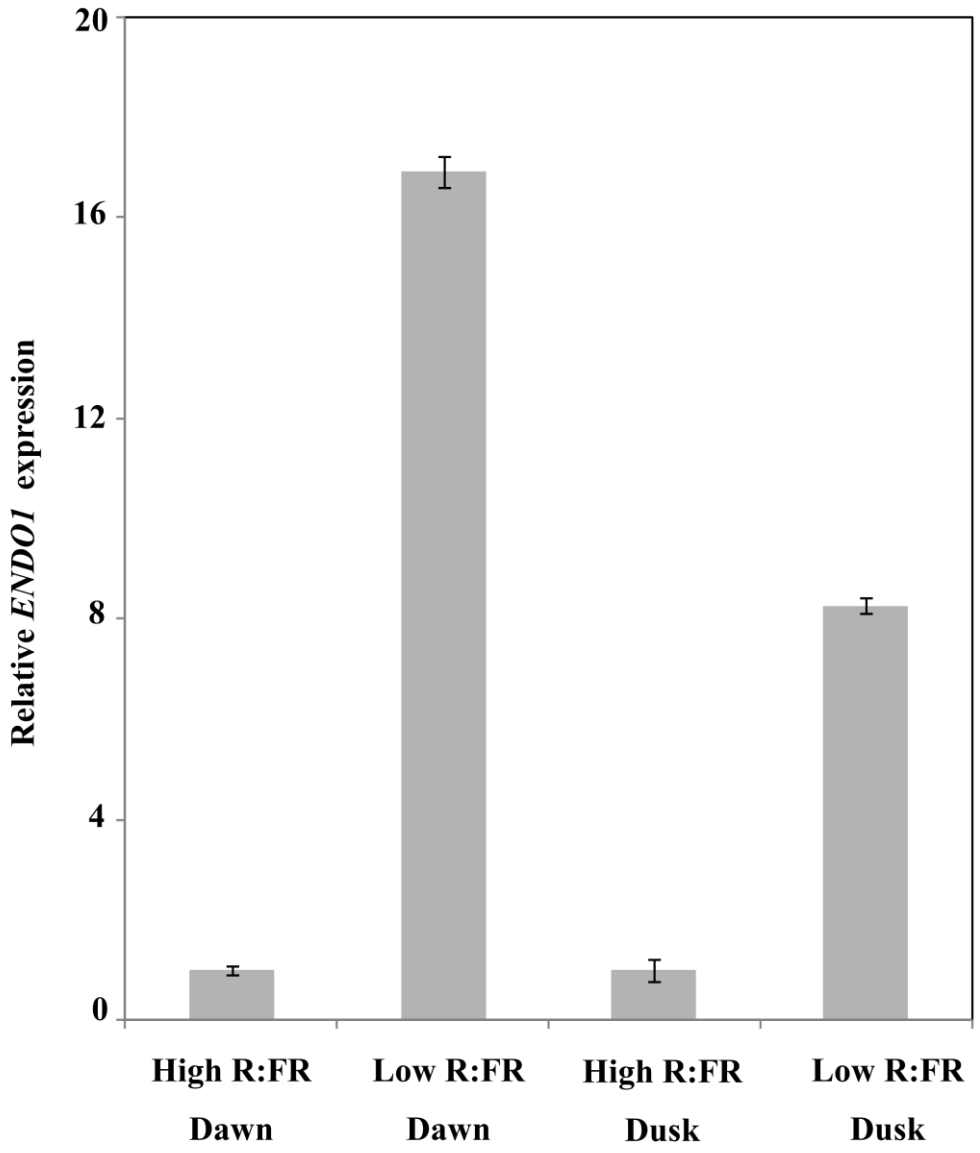


Figure 3.2.1. Analysis of *ENDO1* transcript regulation in 5 day old Columbia seedlings grown at 22°C in response to 1 h low R:FR, compared with the control. The dusk treatment ended 30 min before lights went off. Transcript abundance of *ENDO1* was measured using Quantitative RT-PCR. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalised to *ACTIN2*. Relative transcript is normalised to the high R:FR ratio grown control sample.

3.2.2. Kinetics of ENDO1

Previously, it has been shown that expression of some low R:FR ratio-mediated genes is modulated extremely rapidly by changes light quality conditions. For example, changes in *PIL1* expression can be seen as rapidly as 8 min after onset of reduced R:FR ratio light at dawn (Salter *et al*, 2003). As detailed above, *ENDO1* expression changes within 1 h of low R:FR ratio light treatment, suggesting that it may also respond to this environmental stimuli in a rapid manner (Figure 3.2.1.)

To investigate the kinetics of *ENDO1*expression responses to low R:FR ratio light in more detail, Columbia seedlings were germinated and grown (according to the method in section 2.1.3.) in high R:FR ratio 12 h light/ 12 h dark cycles. After 5 days the seedlings were moved 30 min after dawn to either continuous high R:FR ratio or continuous low R:FR ratio light. At various time points samples were harvested for Quantitative RT-PCR analysis (Section 2.6.1.2., 2.6.2.6.). The results, presented in Figure 3.2.2.a shows that *ENDO1* transcript levels are detectable after only 30 min of low R:FR ratio light. Interestingly after 2 h of low R:FR ratio *ENDO1* transcript abundance drops, suggesting that *ENDO1* is not only upregulated by low R:FR ratio light but that a feedback mechanism regulates the duration of this upregulation. Transcript of *ENDO1* is subsequently seen to increase again after a further 24 h of low R:FR ratio which is suggestive of an influence of the circadian clock on *ENDO1* expression (for further analysis see section 3.5.1.).

To examine the effect of R/FR reversibility on *ENDO1* gene expression, seedlings were grown in accordance to the protocol above, however, in this experiment after 2 h of low R:FR ratio light, the seedlings were returned to high R:FR ratio for a further 30 h. The results of this experiment, shown in Figure 3.2.2.b., demonstrate that *ENDO1* transcript levels fall rapidly after transfer from low R:FR

ratio to high R:FR. When returned to high R:FR ratio, *ENDO1* does not appear to reach the same transcriptional level as when seedlings have remained in high R:FR ratio conditions. This experiment demonstrates that *ENDO1* expression is rapidly regulated by changes in R:FR ratio light. The rapidity of the increase in transcript infers that *ENDO1* may be directly involved in the molecular shade avoidance pathway. In addition the R:FR ratio reversibility indicates that phytochromes play a role in *ENDO1* regulation. In high R:FR ratio light phytochromes B, C, D and E are in their biologically active conformation and one or more of these are likely to be acting to repress *ENDO1* transcription. It is also likely that de-repression occurs through the majority of phytochrome reverting to the Pr confirmation, which can no longer act to repress *ENDO1*.

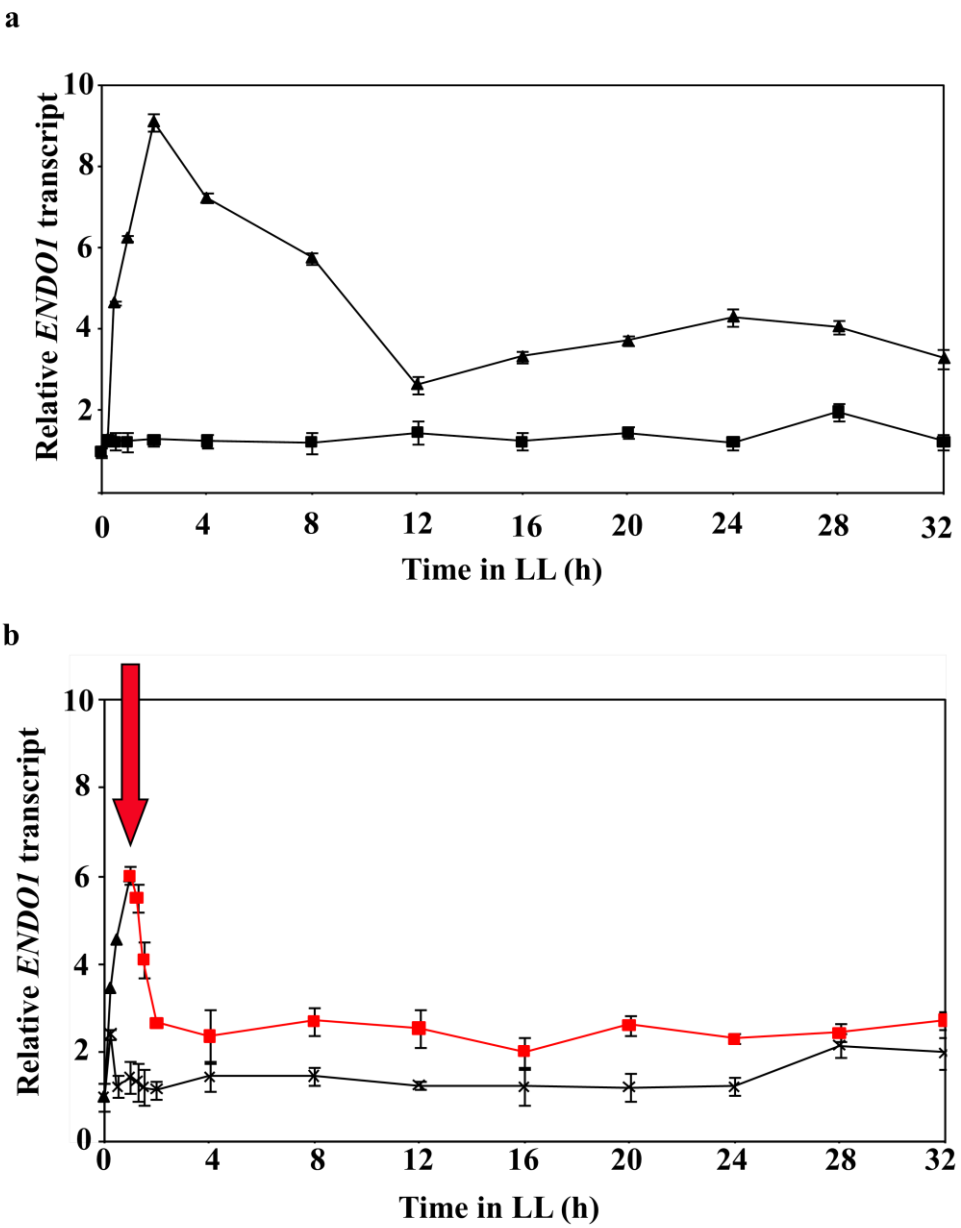


Figure 3.2.2. a Quantitative RT-PCR of *ENDO1* transcript in response to low R:FR ratio in wild-type. a. Rapid time course showing the increase in *ENDO1* transcript abundance in seedlings transferred to continuous low R:FR ratio (▲) and the control seedlings which remained in high R:FR ratio (■). b. Time course of *ENDO1* transcript in seedlings transferred to low R:FR ratio light treatment for 2 h (▲) and then the seedlings were transferred back to high R:FR ratio (red ■) for a further 30 h. The arrow indicates the time point at which the seedlings were moved back into continuous high R:FR ratio. Control seedlings remained in continuous high R:FR ratio (x). Each value is the mean of 3 separate Quantitative RT-PCR reactions normalised to *ACTIN2*. Relative transcript is normalised to the high R:FR ratio grown control sample.

3.2.3. Phytochrome regulation

3.2.3.1. The role of phytochrome B in the regulation of ENDO1

As discussed above, the observation that *ENDO1* transcript is repressed in high R:FR ratio conditions and de-repressed in low R:FR ratio conditions, suggests that *ENDO1* may be regulated by the phytochromes. In order to identify which phytochromes mediate changes in *ENDO1* transcript abundance, phytochrome mutants were studied. Specifically, wild-type and mutant seedlings were grown in 12 h light/ 12 h dark high R:FR ratio light (section 2.1.3.). Subsequently, tissue samples were harvested for Quantitative RT-PCR 1 h after the onset of subjective dawn (section 2.6.1.2. and 2.6.2.6.). Figure 3.2.3.1.a. shows that loss-of function phyB mutants had a higher steady state level of *ENDO1*, thus suggesting that in high R:FR ratio light, phyB acts to repress *ENDO1* transcription. Phytochromes D and E appear to play no additional regulatory role, as *ENDO1* transcript levels were essentially the same in the *phyB* and *phyBDE* mutants. These results are consistent with other molecular and physiological studies showing that responses to low R:FR ratio are primarily mediated by phyB.

3.2.3.2. The role of phytochrome A in the regulation of ENDO1

Phytochrome A is known to act through a molecular pathway which represses the shade avoidance response, thereby limiting the magnitude of this response and promoting survival by preventing exaggerated phenotypes. Given phyA's role in shade avoidance, it was important to determine whether *ENDO1* expression was regulated by this phytochrome.

Experiments were carried out in order to determine whether phyA acts to repress *ENDO1* in low R:FR ratio light. Mutants null for phyA and La-*er* were grown

(see section 2.1.3.) in 12 h light/ 12 h dark light cycles for 5 days, the seedlings were then given 1 h low R:FR ratio commencing 1 h after dawn. Quantitative RT-PCR was subsequently carried out (2.6.1.2., 2.6.2.6.). Figure 3.2.3.1.b. shows that when *phyA* mutants are given transient low R:FR ratio, increased *ENDO1* transcript abundance is seen when compared with that of wild-type seedlings. This observation suggests that phyA plays an important role in repressing *ENDO1* transcript in low R:FR ratio conditions. Taken together, these results suggest that phyA and phyB play antagonistic roles in regulation of *ENDO1* expression under altering R:FR ratio conditions.

It is well known that in response to alterations in ambient light, phytochromes transduce light signals into developmental changes through rapid modulation of transcriptional cascades. The findings presented here clearly show that *ENDO1* is a downstream component of the phytochrome signalling transduction pathway. Specifically my findings suggest that in high R:FR ratio conditions *ENDO1* is repressed principally by the Pfr isoform of phyB. In contrast, in seedlings exposed to low R:FR ratio, phyB is predominantly in its biologically inactive Pr formation, thus resulting in de-repression of *ENDO1*. Additionally, phyA also represses *ENDO1* transcription in response to low R:FR ratio conditions and therefore it is also likely to act to temper the effects of prolonged *ENDO1* expression in low R:FR.

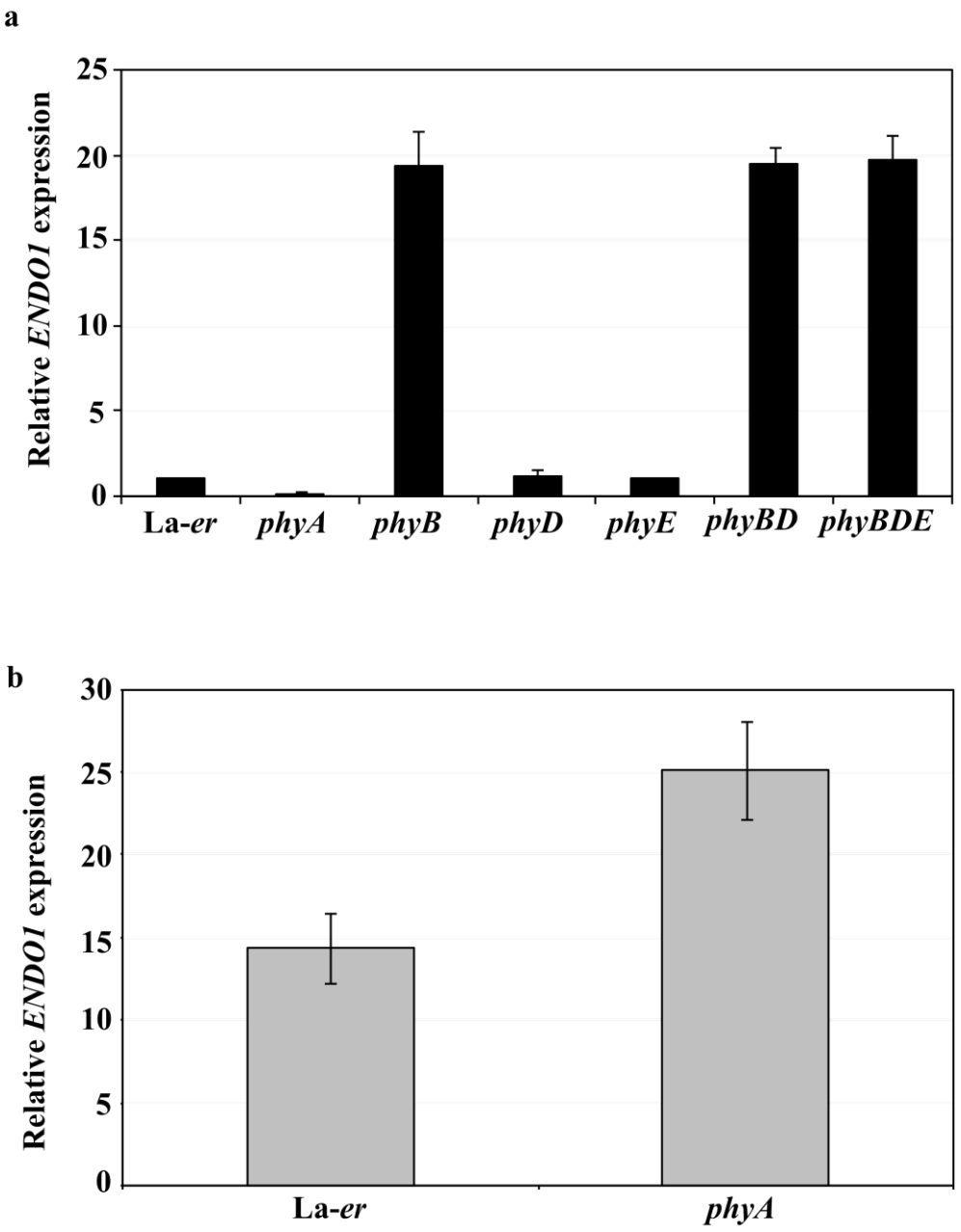


Figure 3.2.3.1. Phytochrome-regulation of *ENDO1* transcript abundance. a. Transcript abundance of *ENDO1* was measured in 5 day old phytochrome mutants grown in 12 h light/ 12 h dark cycles at 22°C, using Quantitative RT-PCR. b. Relative expression of *ENDO1* transcript in response to a 1 h low R:FR ratio light treatment commencing at subjective dawn. Five day old seedlings had been grown in 12 h light/ 12 h dark cycles at 22°C. Relative transcript expression is normalised to *ENDO1* levels in high R:FR ratio grown la-*er* grown plants. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to the wild-type control.

3.2.4. Transcriptional regulators of *ENDO1*

Although much is known about the individual roles of phytochromes as mediators of shade avoidance behaviour, little is known of the underlying molecular mechanisms that transduce phytochrome signals. To date, it has been shown that in its biologically active state phyB translocates to the nucleus and physically interacts with transcription factors. This physical interaction commences the start of a signal transduction cascade and consequently regulates genes involved in the shade avoidance pathway (Nagy and Schaefer, 2000; Quail 2002; Nagatani 2004; Chen *et al*, 2005). The findings within this section suggest that one component of the phyB/ phyA signalling cascade is *ENDO1*. However, components of the cascade that lie downstream of the phytochromes and upstream of *ENDO1* are at present unknown.

One suite of proteins that could target *ENDO1* for regulation are the bHLH transcription factors. The Arabidopsis genome encodes for over 150 putative bHLH transcription factors (Bailey *et al*, 2003). It is known that the bHLH proteins involved in light signalling belong to a single evolutionary related sub-class (Bailey *et al*, 2003; Heim *et al*, 2003; Toledo-Ortiz *et al*, 2003). Of particular interest to the current study are the PIF and PIL bHLH genes as these are known to be involved in the phytochrome-regulated light signalling pathway (Ni *et al*, 1998). Members of the PIF and PIL family control numerous phytochrome responses including seed germination, seedling de-etiolation and shade avoidance (Huq and Quail, 2002; Kim *et al*, 2003; Salter *et al*, 2003; Huq *et al*, 2004; Oh *et al*, 2004; Penfield *et al*, 2005; Lorraine *et al*, 2007). As detailed below, in order to determine whether PIF and PIL family proteins are involved in controlling *ENDO1* expression, Quantitative RT-PCR experiments

were conducted on various Arabidopsis mutant and transgenic lines in which expression of PIF/ PIL family members had been disrupted.

3.2.4.1. Phytochrome interacting factor like 1 R:FR regulation of ENDO1

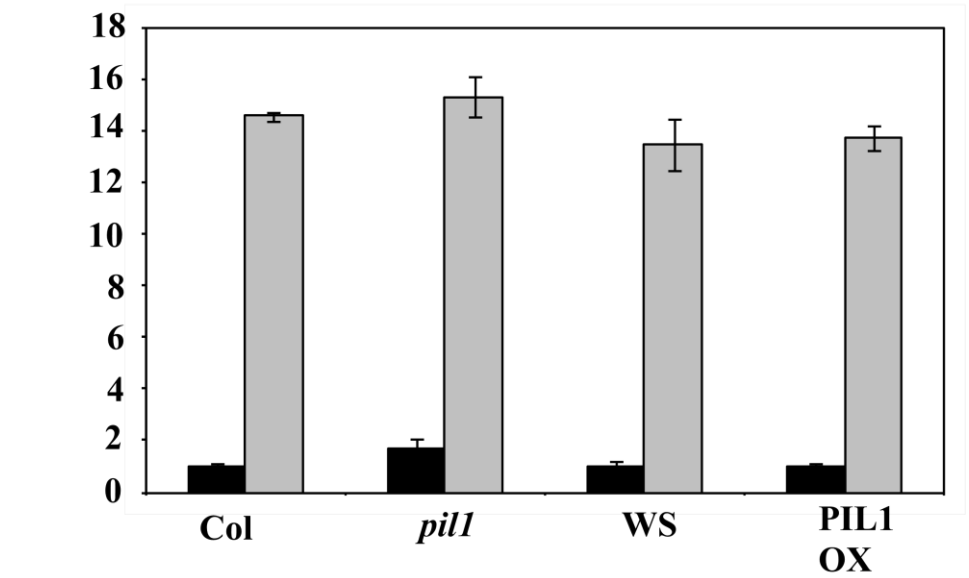
Whilst *PIL1* expression is highly responsive to low R:FR ratio light, it has not been found to directly interact with phyB. However, it is a downstream component of the phy signalling cascade. Recent evidence suggests that *PIL1* contains a G-box motif which is indicative of transcriptional regulation by bHLH proteins. Furthermore, the PIL1 promoter region contains an Ibox motif, a domain which is often strongly associated with light regulation. *PIL1* transcript abundance increases extremely rapidly in response to low R:FR ratio and thus it is conceivable that this transcription factor is involved in controlling expression of other rapidly regulated targets such as *ENDO1*.

To determine whether *ENDO1* is a downstream component of the *PIL1* signalling cascade, *ENDO1* transcript abundance was measured in *pil1* null mutants and over expressing lines. Seedlings were grown for 5 days (section. 2.1.3.) in high R:FR ratio 12 h light/ 12 h dark cycles. On day 6, they were subjected to 1 h low R:FR ratio. Controls remained in high R:FR ratio. Tissue samples were then taken and the results were analysed using Quantitative RT-PCR (Section 2.6.1.2., 2.6.2.6.). Figure 3.2.4.1.a shows that *PIL1* mutants have wild type *ENDO1* transcriptional responses in to altered R:FR ratio. The findings therefore indicates that *PIL1* is not an upstream transcriptional regulator of *ENDO1* in either high or low R:FR ratio conditions.

3.2.4.2. Phytochrome interacting factor 3 R:FR of ENDO1

PIF3 is a known bHLH protein that acts as a primary phytochrome signalling partner (Ni *et al*, 1998). Previous molecular characterisation of *PIF3* demonstrated that it is a G-box binding bHLH protein which interacts preferentially with the active form of phytochrome. *HFR1 (LONG HYPOCOTYL IN FR 1)*, is a known marker gene for shade avoidance, and is known to be a direct target of PIF3 (Fairchild *et al*, 2000).

It is possible that a change in R:FR ratio conditions alters the abundance of phyBPfr: PIF3 complex which in turn, modulate the expression of target genes such as *ENDO1*. To address this question, the relative expression of *ENDO1* in *pif3* null mutants and over-expressing lines exposed to different R:FR ratio light conditions was determined using Quantitative RT-PCR (Section 2.6.1.2., 2.6.2.6.). To do this, seedlings were grown for 5 days (section 2.1.3.) and on day 6 they were subjected to 1 h low R:FR ratio, whilst controls remained in high R:FR ratio. Figure 3.2.4.1.b demonstrates that in wild-type seedlings, *ENDO1* shows an increase in relative expression in response to low R:FR ratio, and that this response is maintained in both *pif3* null mutant and over-expressing lines. This implies that, like *PIL1*, PIF3 does not play a role in R:FR ratio light mediated changes in transcriptional regulation of *ENDO1*.



b

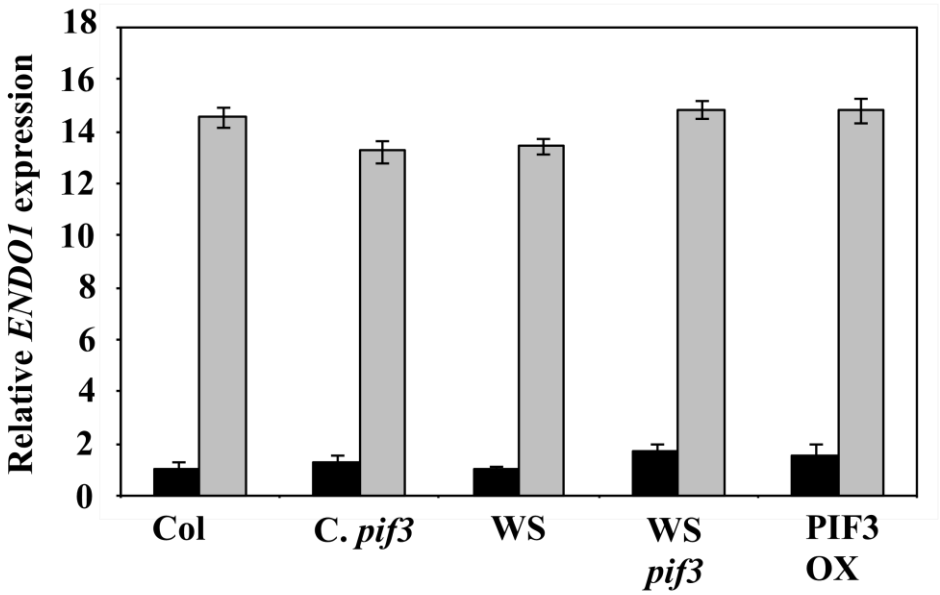


Figure 3.2.4.1. a. Transcript abundance of *ENDO1* was measured using Quantitative RT-PCR in 5 day-old *pil1* mutants and over-expressers in response to 1 h low R:FR ratio treatment at dawn (gray bars). Controls remained in high R:FR ratio (black bars). b. Transcript abundance of *ENDO1* was measured using Quantitative RT-PCR in 5 day-old *pif3* mutants and over-expressers in response to 1 h low R:FR ratio treatment at dawn (gray bars). Controls remained in high R:FR ratio (black bars). All seedlings were grown in 12 h light /12 h dark cycles at 22°C. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalised to *ACTIN2*. Relative transcript abundance is normalised to the high R:FR ratio grown wild-type sample.

3.2.4.3. R:FR ratio regulation of SHL

Previous work has demonstrated that *SHL* (SHORT LIFE) may regulate *ENDO1* transcription (Mussig and Altmann, 2003). Expression profiles showed that in a 35S::*SHL* plant an increase in *ENDO1* transcript was seen, strongly suggesting that SHL positively regulates *ENDO1* transcription (Mussig and Altmann, 2003). It has also been shown that SHL is necessary for normal plant development, plants over expressing SHL show retarded growth and early flowering (Mussig and Altmann, 2003). The nuclear protein SHL contains a PHD (Plant Homeodomain) and a BAH (Bromo-Adjacent Homology) domain, both of which are thought to be involved with protein–protein interactions that are associated with transcriptional regulation and chromatin remodelling.

As a preliminary study to examine whether *SHL* is also regulated by a change in R:FR ratio, wild-type seedlings were grown for 5 days (section 2.1.3.) in 12 h light/ 12 h dark conditions, on day 6 they were subjected to 1 h low R:FR ratio, whilst controls remained in high R:FR ratio. Interestingly, the resulting Quantitative RT-PCR (Section 2.6.1.2., 2.6.2.6.) suggests that *SHL* may be regulated by changes in R:FR ratio. However, experimental results varied: On two occasions an increase in transcript was seen after only 1 h of low R:FR ratio (Figure 3.2.4.3. a and b) whereas on the third, little change was observed (Figure 3.2.4.3 b). Further investigations are therefore required to determine whether *SHL* has a role in shade avoidance signalling.

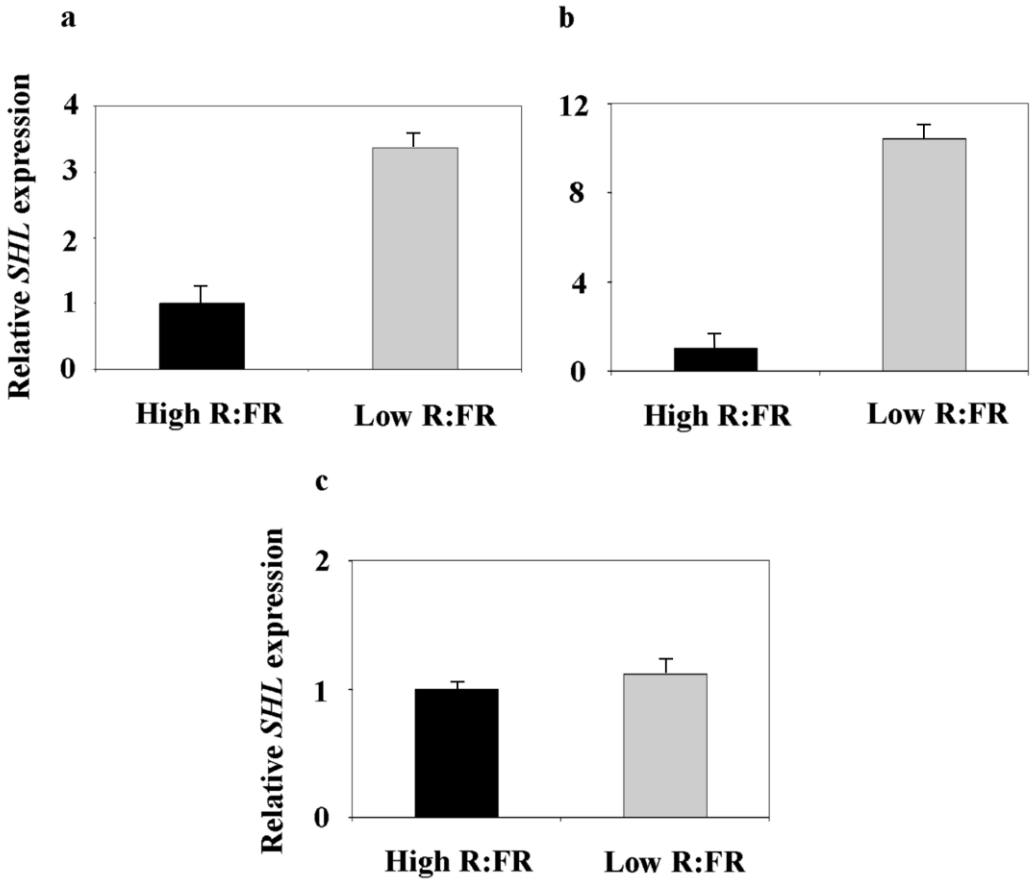


Figure 3.2.4.3. Quantitative RT-PCR analysis of *SHL* transcript levels following a 1 h treatment of low R:FR ratio at dawn (3 biological repeats (a. b. And c.)). The wild-type seedlings were 5-days old grown in 12 h light/ 12 h dark at 22°C. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalized to *ACTIN2*. Relative transcript abundance is normalised to wild-type high R:FR ratio sample.

3.2.5. Circadian regulation of *ENDO1*

A growing body of evidence suggests that certain low R:FR ratio light regulated genes and physiological responses are modulated by the circadian clock. For example, whilst an increase in *PIL1* transcript reliably occurs in response to a transient 30 min low R:FR ratio pulse, the magnitude of this response varies markedly with the time of day (Salter *et al*, 2003). The circadian cycling of *PIL1* transcript abundance is observed in low, but not high, R:FR ratio light conditions. Transcript of *PIL1* peaks at around subjective dawn and subsequently declines to minimum levels towards the end of the subjective day. This cycle persists through to the second day.

The oligoarray data in which *ENDO1* was initially discovered to respond to low R:FR ratio also showed that the magnitude of the *ENDO1* transcriptional response varied at different time points throughout the day (Salter *et al*, 2003). *ENDO1* showed a peak 8.4 fold increase in transcript in response to a 1 h low R:FR ratio pulse at subjective dawn on day one, and this peak in transcript was seen again 24 h later. Similar to *PIL1*, no marked changes were seen in *ENDO1* transcription following high R:FR ratio treatment.

To extend this work, *ENDO1* transcriptional responses to 1 h low R:FR light at both subjective dawn and subjective dusk were analysed (for experimental detail see section 3.2.1.1). As shown in figure 3.2.1.1. This demonstrated that whilst *ENDO1* transcription is responsive to low R:FR treatment at both dawn and dusk, sensitivity to low R:FR ratio peaks at dawn.

To confirm that *ENDO1* is under the control of the circadian clock, wild-type plants were grown for 21 days in 12 h light/ 12 h dark high R:FR ratio cycles (section 2.1.3.). Commencing at the onset of subjective dawn on day 22 the plants were moved

into continuous high R:FR ratio conditions and at various time points were given a 1 h pulse of low R:FR ratio. Quantitative RT-PCR was then carried out (Section 2.6.1.2., 2.6.2.6.). Figure 3.2.5. clearly shows that in transient low R:FR ratio conditions, *ENDO1* transcription follows a circadian rhythm. The results also show that the initial transient low R:FR ratio light pulse causes a marked 16-fold increase in *ENDO1* transcript, whereas the transient pulse given towards the middle/ end of the day only causes an 8-fold increase in transcript. However, at subjective dawn on the second day a marked (16 fold) change in transcript is seen again. No clear changes in *ENDO1* transcript can be seen in response to high R:FR ratio.

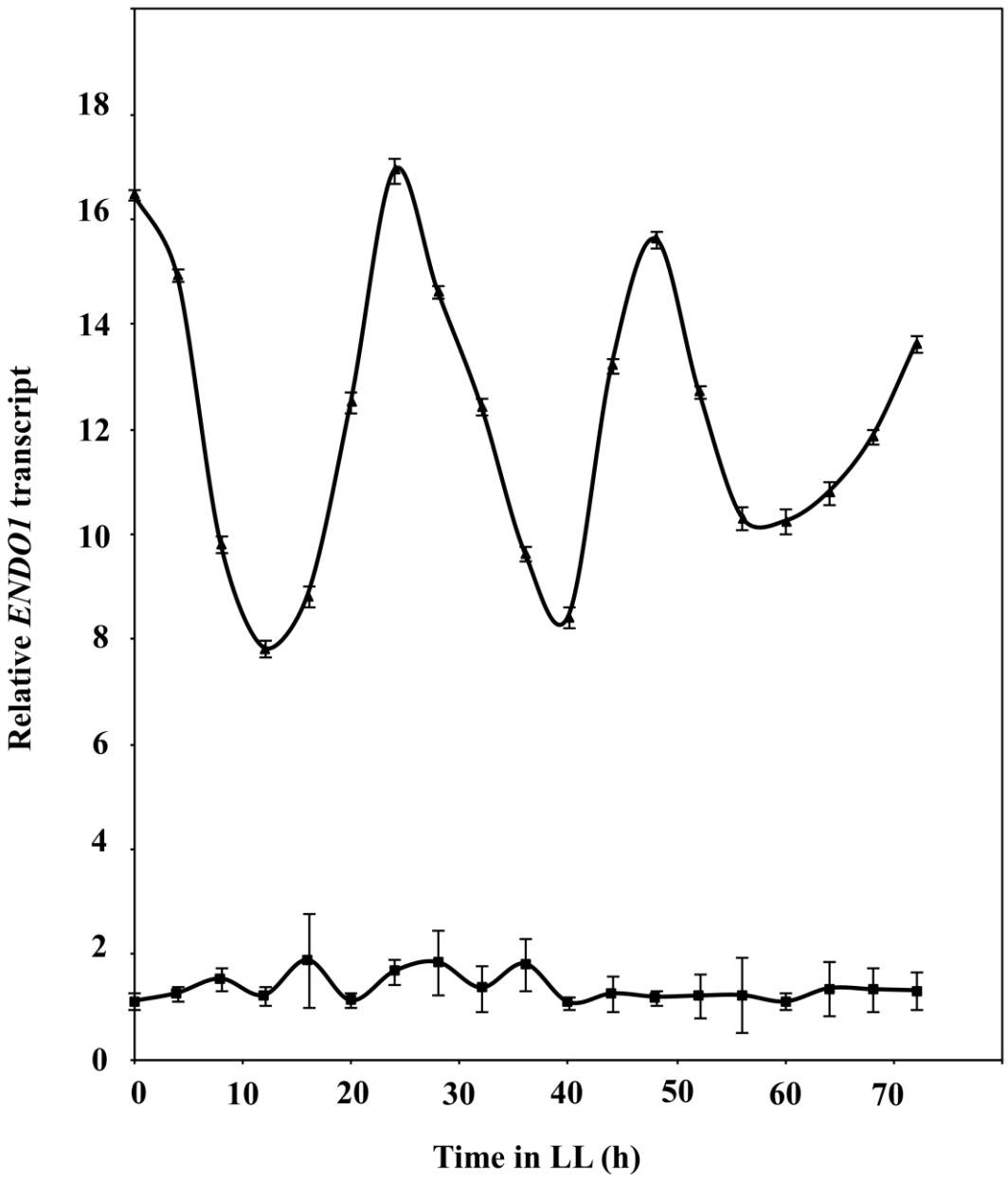


Figure 3.2.5. Circadian analysis of *ENDO1* transcript regulation by low R:FR ratio (▲) at 22°C in wild-type plants. Controls remained in high R:FR ratio (■). Plants were grown in 12 h light/ 12 h dark for 21 days, on day 22 they received the various treatments. Transcript abundance of *ENDO1* was measured using Quantitative RT-PCR. Each value is the mean of 3 separate reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to the high R:FR ratio grown sample at time 0.

3.2.6. R:FR ratio regulation of At2g43590, AtEP3 At2g43610 and At2g43620

There are a total of 9 Arabidopsis class IV chitinases however, 4 of these are thought to be pseudogenes as they lack essential amino acid residues required for enzymatic function (Passarinho and de Vries S, 2002). Of those that produce functional protein products At2g43590, AtEP3/Atchit IV, At2g43610 and At2g43620 show sequence homology to *ENDO1*. Thus it is possible that they are regulated in a light-dependent fashion similar to *ENDO1* and may, collectively or separately, play a role in the shade avoidance pathway. Alternatively these genes may be light-independent and play an entirely different role in plant development.

To determine whether these other class IV chitinases are responsive to changes in R:FR an experiment was set up using wild-type seedlings. The seedlings were grown (as detailed in section 2.1.3.) in high R:FR ratio 12 h light/ 12 h dark for 5 days. At subjective dawn on day 6 the seedlings were given a 1 h transient low R:FR ratio pulse, tissue samples were then taken and subjected to Quantitative RT-PCR (section 2.5.1.2. and 2.5.2.6.). The results shown in Figure 3.2.6. indicate that none of these 4 chitinase-like genes (At2g43590, AtEP3/Atchit IV, At2g43610 and At2g43620) respond in any significant manner to a change in R:FR ratio; their transcript levels remain approximately the same in both high R:FR ratio and low R:FR ratio conditions. This suggests that *ENDO1* is the only chitinase of the 5 functional class IV chitinases studied to show alterations in transcript in response to a 1 h change in light quality at dawn.

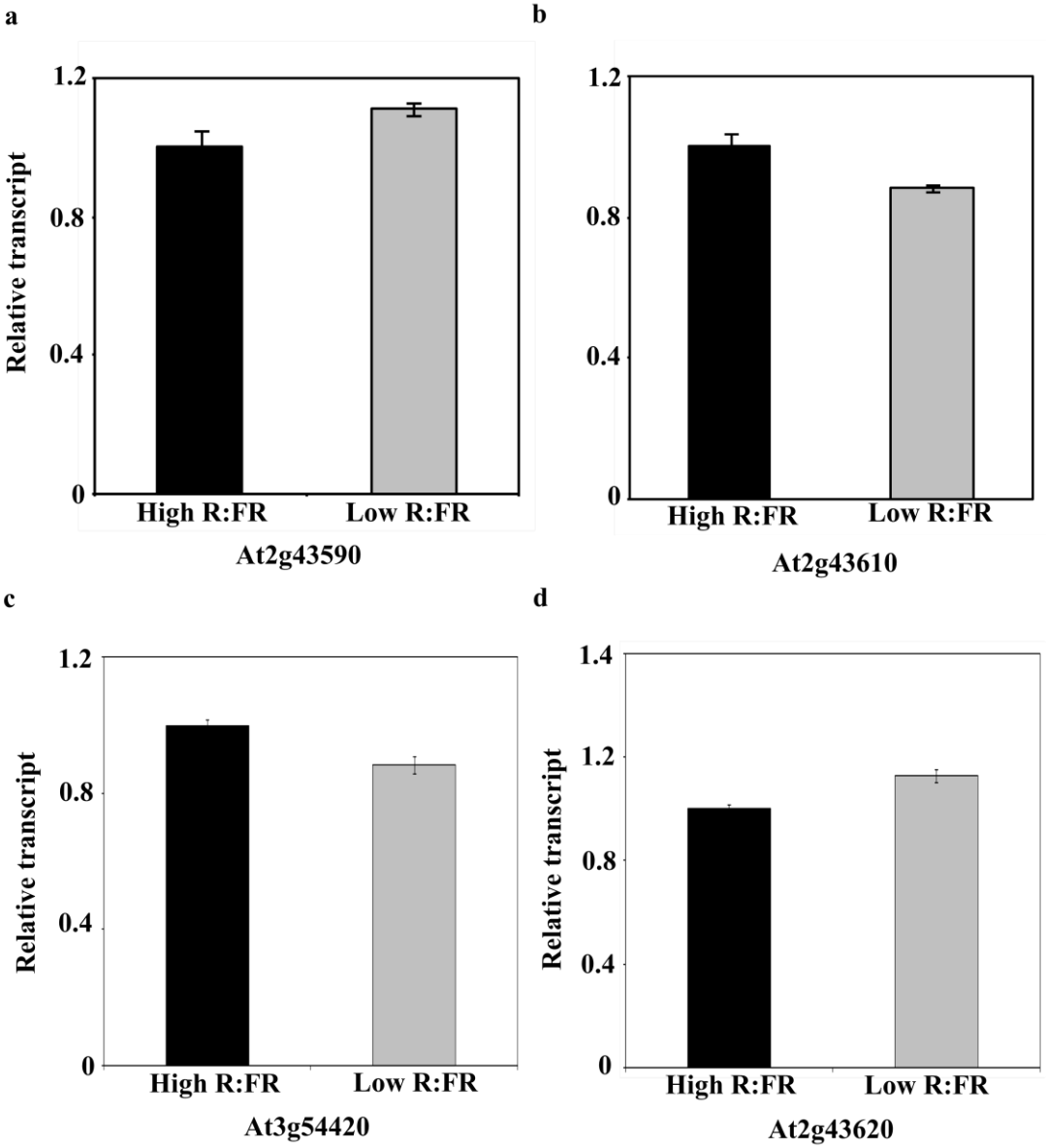


Figure 3.2.6. Quantitative RT-PCR analysis of class IV chitinases transcript levels following a 1 h treatment of low R:FR ratio at dawn. The wild-type seedlings were 5-days old grown in 12 h light/ 12 h dark at 22°C. Each value is the mean of 3 separate quantitative RT-PCR reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to wild-type high R:FR ratio sample.

3.2.7. Biological function of ENDO1

It is well known that plant development is strongly influenced by the ambient light environment and this plasticity permits appropriate adjustments in growth behaviour. Shade avoidance has a suite of developmental responses and phyB is known to play a predominant role control of this process. There are both short and long term physiological responses to changes in R:FR ratio (Franklin and Whitelam 2006). Certain physiological responses, such as hypocotyl and petiole elongation, are extremely rapid whilst others, such as acceleration of flowering, occur only when plants have been subjected to prolonged low R:FR ratio. Since *ENDO1* expression appears to be rapidly modulated by changes in R:FR light, it is possible this gene is a component of the signalling pathways that elicit short term changes to this environmental stimuli. The following sections discuss the effects of perturbing *ENDO1* expression on short and long term physiological responses to simulated shade conditions.

3.2.7.1. T-DNA insertion lines; endo1-1 and endo1-2

To analyse a physiological role for *ENDO1* in shade avoidance, two *ENDO1* null lines were obtained. The first independent line homozygous for the T-DNA insertion was obtained (endo1-1) from the Nottingham Arabidopsis stock centre and the second mutant knockout line (endo1-2), was obtained from the Institut National de la Recherche Agronomique (INRA) France. Primers were designed to target the T-DNA sequence, allowing amplification of this region (see section 2.6.2.4.) and subsequent determination of homozygous lines (figure 3.2.7.1.a and b). RT-PCR analysis also failed to detect any *ENDO1* transcript see figure 3.2.7.1.c and d. The transgenic lines

were also sequenced (section 2.6.2.7.) to determine the precise location of the T-DNA insertion (figure 3.2.7.1.) Once *endo1* mutants had been isolated, physiological experiments were conducted to determine whether these lines displayed an aberrant response to low R:FR ratio light.

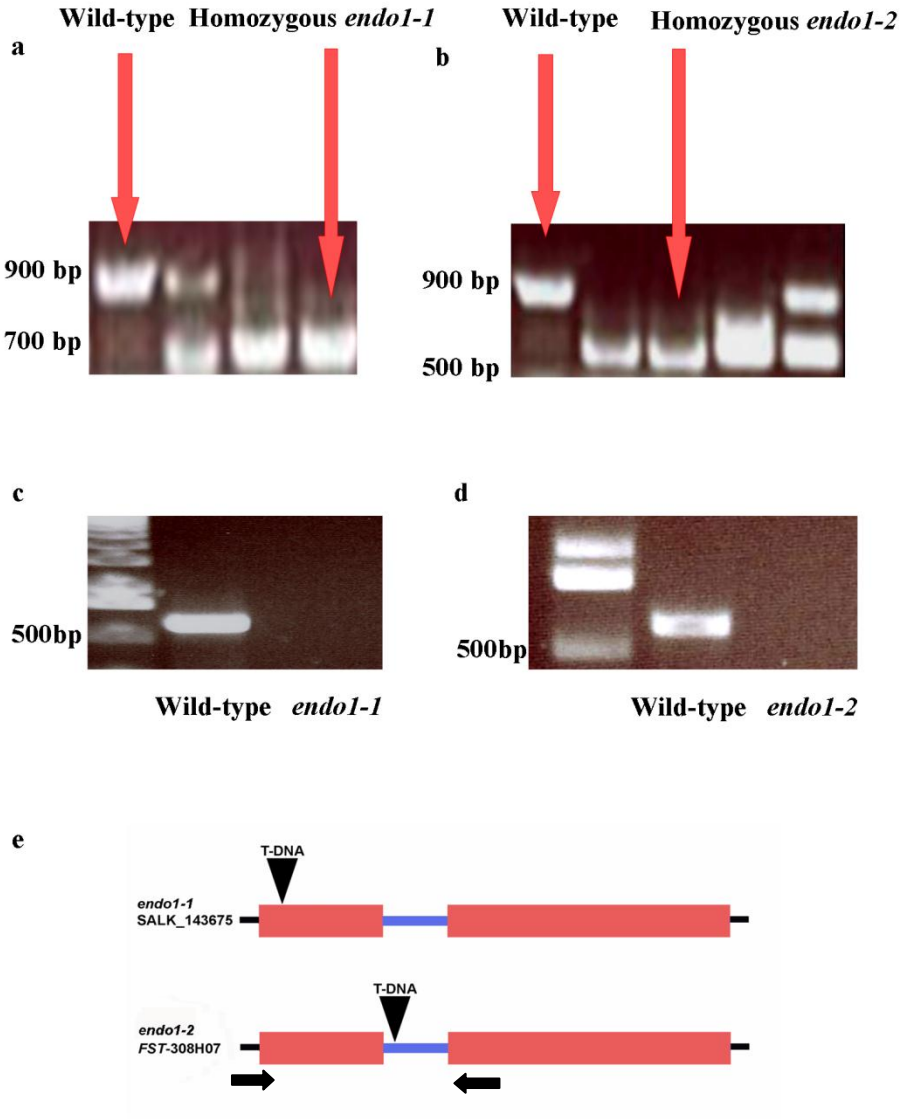


Figure 3.2.7.1. a and b. Genomic primers were designed and RT-PCR was carried out identifying the presence of the T-DNA insert in *endo1-1* and *endo1-2* lines. c and d. RT-PCR detecting no transcript in *endo1-1* and *endo1-2* mutants. e. The position of the T-DNA inserts in the *endo1* mutants. The forward and reverse arrows represent the forward and reverse primers used for RT-PCR verification.

3.2.7.2. The effect of low R:FR ratio on hypocotyl elongation in endo1 mutants

One of the most striking shade avoidance responses is enhancement of hypocotyl elongation. It is known that a single, 2 h transient low R:FR ratio pulse at subjective dusk is sufficient to induce a significant promotion of hypocotyl growth in seedlings, whereas a similar treatment at subjective dawn leads to an inhibition of hypocotyl elongation. This hypocotyl response has now been found to be due to continuous light conditions however, when seedlings are grown in light dark cycles, maximum elongation growth occurs early in the morning (Lorraine *et al*, 2007).

In order to test whether *ENDO1* is required for circadian regulated hypocotyl elongation responses to occur, an experiment was set up whereby seedlings were grown (section 2.1.3.) for 5 days in 12 h light/ 12 h dark high R:FR ratio. On day 6 seedlings were given a 2 h pulse of low R:FR ratio which ended 30 min before the onset of dusk, the controls remained in high R:FR ratio (section 2.2.2.). The seedlings were then measured 24 h later using ImageJ (see section 2.2.6.). Figure 3.2.7.2. shows that in both mutant and wild-type seedlings, a low R:FR ratio treatment leads to an equivalent *ca.* 20% promotion of growth at dusk. Thus, *ENDO1* does not appear to be an essential molecular component of hypocotyl elongation growth machinery.

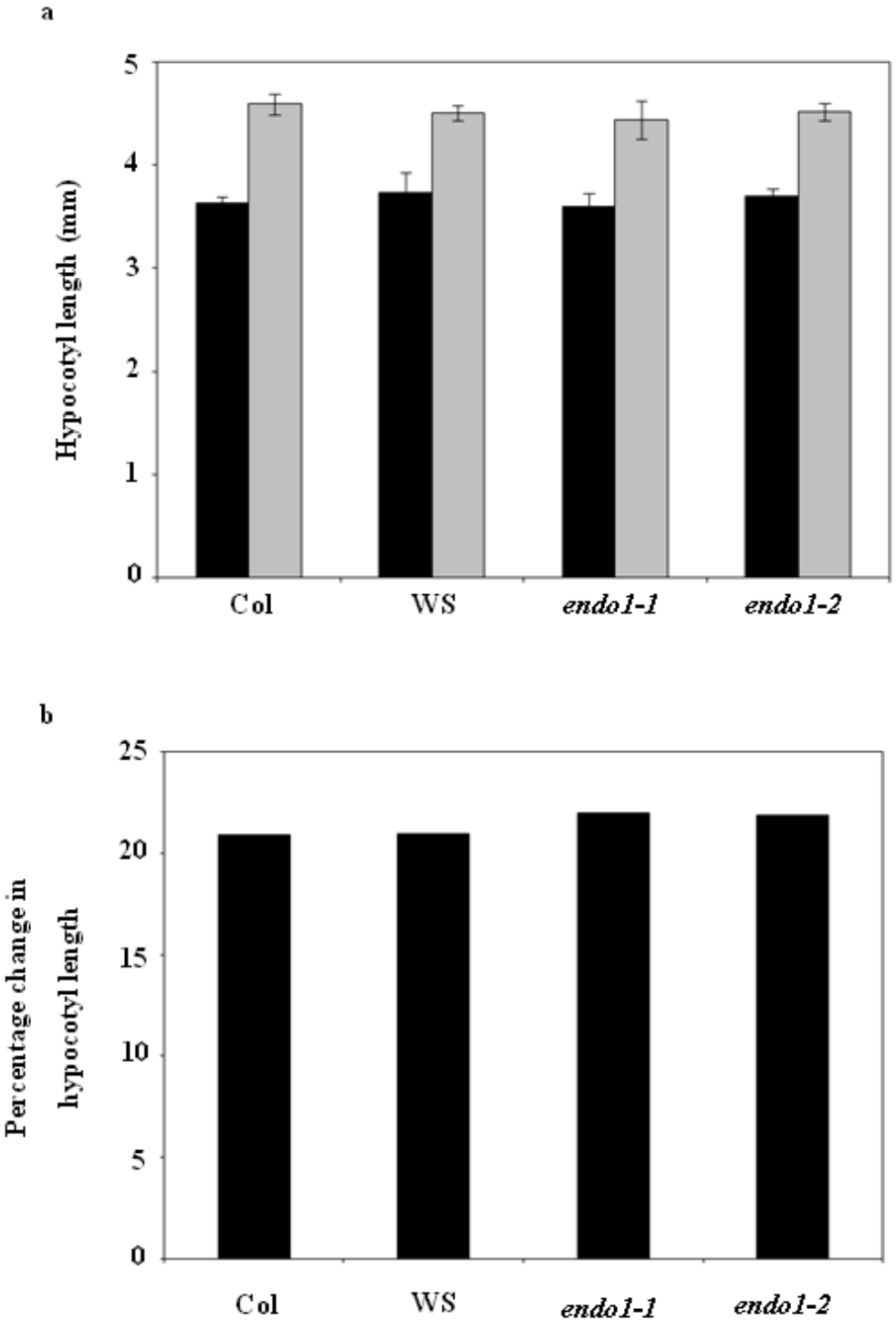


Figure 3.2.7.2. a. Hypocotyl lengths of the relevant wild-types and mutant *endo1* plants, grown for 5 days in 12 h light/ 12 h dark cycles at 22°C. On day 6 seedlings were treated with a single 2 h reduction in R:FR ratio (grey bars) ending 30 min before the end of the light period. Controls remained in high R:FR ratio (black bars). Hypocotyls were measured 24 h after the end of the treatment. Error bars represent SE. b. Shows percentage change in hypocotyl lengths. A sample size of 15 was used for each genotype.

3.2.7.3. The effect of low R:FR ratio on petiole length in endo1 mutants

The elongation of Arabidopsis petioles is a phytochrome-mediated shade avoidance response (Franklin *et al*, 2003). In particular, phyB is actively involved in petiole elongation responses to changing R:FR ratio conditions. As previous results in this chapter demonstrate *ENDO1* transcriptional regulation by phyB, it is possible that *ENDO1* has a role in the low R:FR ratio mediated petiole elongation response.

To determine whether this was the case, wild-type and *endo1* mutant plants were grown according to section 2.1.3. for 14 days in high R:FR ratio 12 light/ 12 h dark, they were then moved to low R:FR ratio for a further 7 days (section 2.2.4.). Controls remained in high R:FR ratio. The petiole length of leaf 5 was measured (according to section 2.2.7.) from a total of 15 plants from each treatment. The results show (figure 3.2.7.3.) that the wild type plants show an *ca.* 30% increase in petiole length in response to low R:FR ratio. The *endo1* mutants however, appear to show a reduced increase in petiole elongation (*ca*. 10%) in response to low R:FR ratio. It is also important to note that in high R:FR ratio *endo1-1* and *endo1-2* have slightly longer petioles when compared to wild type. These findings suggest that ENDO1 is a component necessary for the petiole growth signalling pathway.

3.2.7.4. The effect of low R:FR ratio on Flowering time in endo1 mutants

In Arabidopsis flowering is accelerated by a prolonged reduction in R:FR ratio which acts to signal the presence of neighbouring vegetation. Such a strategy is a crucial component of competition in higher plants. To identify whether *ENDO1* is required for the transition from vegetative growth to low R:FR ratio induced flowering wild-type plants, *endo1-1* and *endo1-2* mutants were germinated (2.1.3.) and then grown in 12 h light / 12 h dark high R:FR ratio for 7 days. The seedlings were then transferred

to soil trays (2.2.4.) and remained in either high R:FR ratio 12 h light / 12 h dark or were moved to low R:FR ratio 12 h light/ 12 h dark. The plants remained in these conditions until the onset of bolting. Flowering time in this experiment was measured by the number of rosette leaves on each individual plant. Plants subjected to prolonged low R:FR ratio show hastened flowering when compared to plants that remained in high R:FR ratio. The results show (figure 3.2.7.4.) that *endo1* mutants flower with approximately the same number of leaves as wild-type, signifying that *ENDO1* plays no observable role in the acceleration of flowering in response to low R:FR ratio.

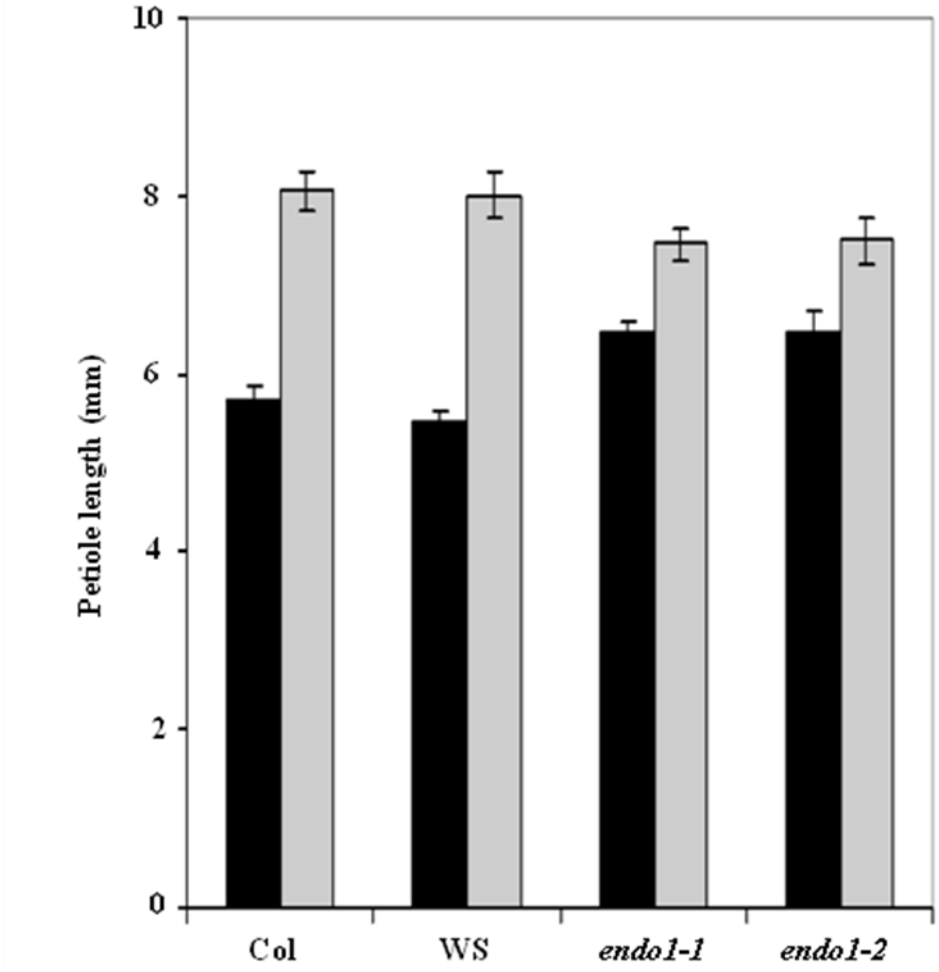


Figure 3.2.7.3. a. Petiole length of relevant wild-types and mutant *endo1* plants, grown for 7 days in high R:FR ratio 12 light/ 12 h dark. They were then moved to low R:FR ratio for a further 7 days (grey bars). Controls remained in high R:FR ratio light dark cycles (black bars). Petiole length was measured at the end of the 7 day treatment. A sample size of 15 was used for each genotype. Error bars represent SE.

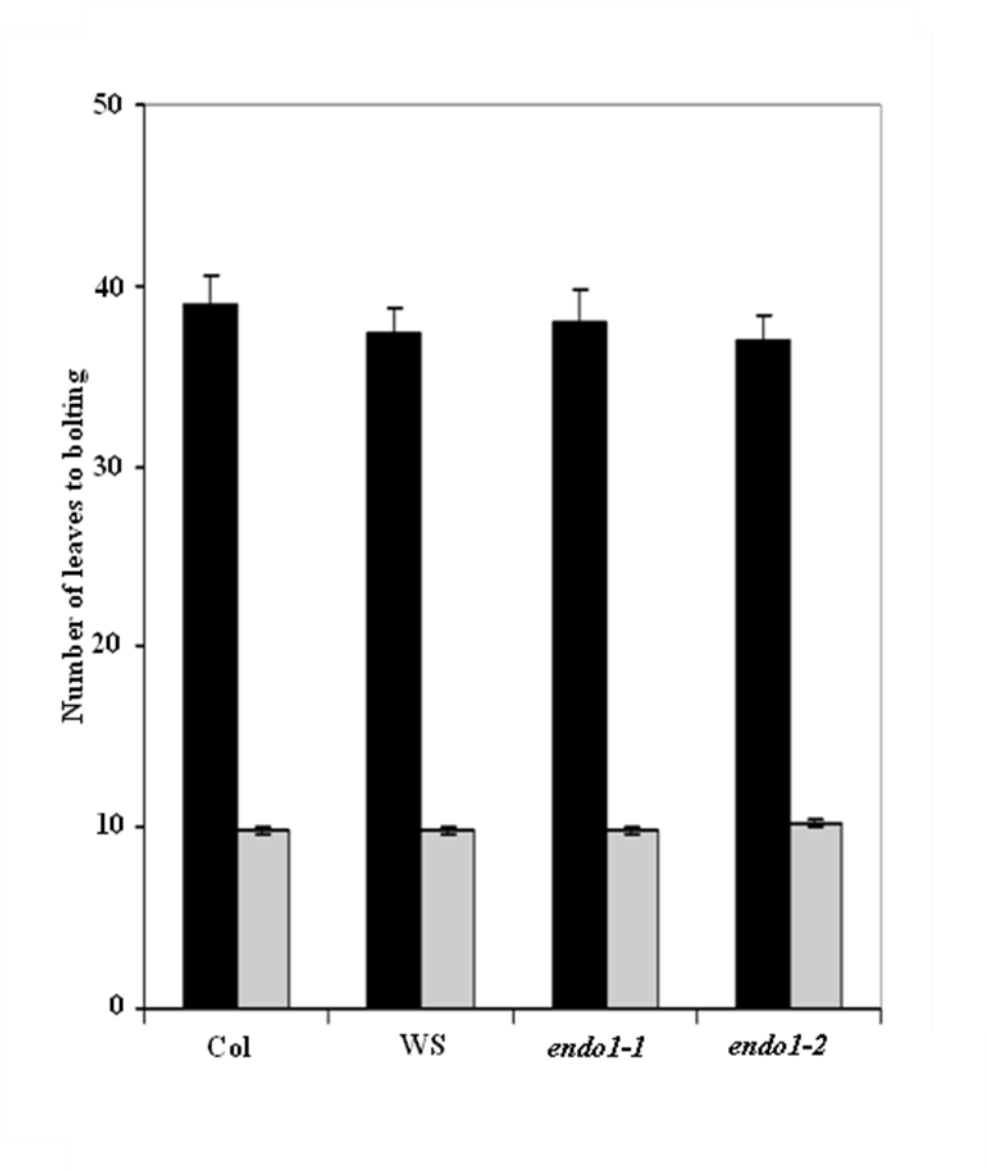


Figure 3.2.7.4. a. Wild-type and *endo1* mutants were grown in 12 h light/ 12 h dark for 7 days. The seedlings were then moved to 12 h light/ 12 h dark low R:FR ratio conditions (grey bars) or they remained in high R:FR ratio conditions until bolting (black bars). The number of rosette leaves were then counted at the onset of bolting. A sample size of 15 was used for each genotype. Error bars represent SE.

3.2.8. Identification of promoter motifs

To identify other possible transcriptional regulators of *ENDO1*, the gene was examined for conserved sequence motifs in its promoter. This was performed using the PLACE Web Signal Scan Program. This program identifies previously described motifs within the promoter region. All light regulation related promoter motifs and circadian related promoter motifs which have been identified, are present in the table below.

Elements and related motifs	Sequence	Description of motif
-10PEHVPSBD	TATTCT	Promoter element found in barley involved in circadian rhythms and light regulation
ASAMOTIFCAMV	GACG	ASF-1 binding site in CAM35S promoter. GACG motifs are found in many promoters and are involved in transcriptional activation of several genes by auxin salicylic acid. May be relevant to light regulation
GTI CONSENSUS	GRWAAW	Consensus GT-1 binding site in many light regulated genes
IBOX	GATAAG	Conserved sequence upstream of light regulated genes binding site of MYB1-like proteins, MYB1 acts as a transcriptional activator
REALPHALGLHCB21	AACCAA	REalpha found in lemna gibba lhcb 21 gene promoter required for phy regulation
SoriPIAT	GCCAC	One of the sequences over represented in light induced promoters (SORLIPS) in Arabidopsis

Table 3.2.8. Light/ circadian regulatory motifs found in 1000 bp of *ENDO1* promoter region. Promoter elements found using Web Signal Scan Program (PLACE).

3.3 Discussion

These results demonstrate that *ENDO1* is upregulated during shade avoidance. Additionally, this upregulation is rapid, occurring after only a 30 min reduction in R:FR ratio. Furthermore, a robust circadian regulation of *ENDO1* in response to low R:FR ratio has been identified, with maximal de-repression of *ENDO1* occurring at subjective dawn. Phytochrome B appears to be the principal phytochrome acting to repress *ENDO1* in high R:FR ratio light conditions. In an opposite manner, phyA represses *ENDO1* in low R:FR ratio conditions, perhaps acting antagonistically with phyB to prevent excessive induction of *ENDO1* transcript. The phytochromes function as integral light switchable components of transcriptional regulatory complexes. It is therefore probable that known light-regulated transcription factors are directly involved in controlling the timing of *ENDO1* expression. However, an upstream known light-regulated transcription factor of *ENDO1* was not identified in the current study. Despite this, the findings in this chapter allow the exclusion of PIL1 and PIF3 as transcriptional regulators of *ENDO1*. The PIF transcription factors are known to recognise and bind to DNA promoter domains that contain a G-box motif, since the promoter region of *ENDO1* lacks a G-box binding domain it is unlikely that the PIFs directly interact with *ENDO1*. Thus it is more likely that downstream targets of the PIFs act as transcriptional activators of *ENDO1*. However, it is still possible that other PIF members; such as PIF4 and PIF5 regulate *ENDO1* transcription indirectly, and future studies will help determine whether this is the case.

Preliminary data implicates SHL as a putative R:FR ratio mediated positive regulator of *ENDO1* transcription (Mussig and Altmann, 2003), although again additional work is needed to confirm whether SHL is in fact a member of the

molecular shade avoidance pathway. In conclusion, the transcriptional enhancers of the *ENDO1* gene remain elusive at present.

ENDO1 is known to respond to a number of stresses such as water deficit (Bray, 2004), high salinity (Takahashi *et al*, 2004) and oxidative stress (Takahashi *et al*, 2004). An alternative explanation for the increase in *ENDO1* transcript in response to a decrease in R:FR ratio is that a change in light quality could be acting as an abiotic stress to plants. If this were the case then *ENDO1* may be a gene which is up-regulated when plants are exposed to a specific stressor. However this is unlikely as one would expect a great many stress-related genes to have identified as R:FR ratio responsive. Whilst it is known that a small number of stress responsive genes such as *COR15* and *KIN1* do respond to changes in light quality, the majority do not (Cao *et al*, 2005).

The observation that petiole elongation is reduced in *endo1-1* and *endo1-2* mutant plants exposed to low R:FR ratio light provides evidence that ENDO1 modulates cell wall growth. As previously discussed (see Introduction), chitinase-like genes show homology to the Cowpea cell wall growth yeildin genes and these proteins are known to be involved in the regulation of growth. It is therefore tempting to suggest that in reduced R:FR ratio environments ENDO1 contributes to cell wall restructuring events during petiole elongation. However, my finding that *endo1* mutants have a retarded petiole elongation response, suggests that if this is indeed the case, it acts redundantly with other cell wall modulatory genes. Thus whilst my findings do not exclude a possible role for ENDO1 in other cell wall growth and shade avoidance responses, such as hypocotyl elongation, they do suggest that if ENDO1 is involved in these processes, it is through a complex signalling pathway involving genes that act in a redundant fashion.

4. *DIN2*

4.1. Introduction

DARK INDUCIBLE (DIN) genes were initially identified in Arabidopsis and radish (Azumi and Watanabe, 1991; Fujiki *et al*, 2000a; Fujiki *et al*, 2001). The *DIN* genes were found to show elevated transcript abundance following transfer of intact plants or detached leaves to darkness (Azumi and Watananbe, 1991; Fujiki *et al*, 2000b; Fujiki *et al*, 2001). Other than being dark inducible, the *DIN* genes have little in common, coding for a diverse array of functionally dissimilar proteins. As it is known that darkness causes a dramatic acceleration in leaf senescence (Nooden 1988; Azumi and Watanabe, 1991; Quirino *et al*, 2000) through protein and chlorophyll degradation (Nooden, 1988), it was suggested that *DIN* genes act as mediators of the leaf senescence process (Fujiki *et al*, 2001).

Interestingly, the timing of expression of the Arabidopsis *DIN* gene family varies significantly according to the duration of dark exposure: *DIN* genes can be subdivided into two distinct groups termed early and late responsive (Fujiki *et al*, 2000). *DIN1*, *DIN3*, *DIN4*, *DIN6* and *DIN10*, members of the early responsive family, show detectable transcript levels as early as 3 h after the onset of dark treatment. By contrast, *DIN2*, *DIN9* and *DIN11*, members of the late responsive family, generate transcript after more prolonged (24 h) dark treatment (Fujiki *et al*, 2000 and Fujiki *et al*, 2001). The main aim of this chapter is to determine whether one specific *DIN* gene; *DIN2*; is also a component of the shade avoidance signalling pathway.

4.1.1. Dark inducible 2; a member of glycoside hydrolase family 1

DARK INDUCIBLE 2 is a member of glycoside hydrolase family 1 that encodes a protein similar to a β-glucosidase. The Arabidopsis genome has 48 putative β-

glucosidase genes of which are 8 probable pseudogenes. With the exception of At3g06510; *SFR2* which belongs to a distinct lineage, all members of the β-glucosidase family share a common evolutionary origin and have been subdivided into approximately 10 subfamilies. β-glucosidases hydrolyze either *O*-linked β-glycosidic bonds (β-D-glucoside glucohydrolase) or *S*-linked β-glycosidic bonds (myrosine or β-D-thioglucoside glucohydrolase), thereby liberating glucose from glucose polymers. In higher plants these enzymes have been implicated in processes such as chemical defence against pathogens (Niemeyer, 1988), phytohormone activation (Brzobohaty *et al*, 1993), lignification (Dharmawardhana *et al*, 1995) and cell wall catabolism (Leah *et al*, 1995). Additionally, SFR2 (Sensitive to Freezing 2) has recently been discovered to encode a protein homologous to family 1 β-glucosidase and has been found to be essential for freezing tolerance (Thorlby *et al*, 2004).

4.1.2. Dark inducible expression is controlled by cellular sugar levels

Dark inducible 2 is a member of the late responsive β-glucosidase gene family. A growing body of evidence indicates that *DIN* gene expression is controlled by cellular sugar levels. Fujiki and colleagues (2001) demonstrated that detached leaves exposed to darkness and floated on sugar solution did not synthesize *DIN2* transcript. Fujiki *et al*, (2001) also found that the application of a photosynthetic inhibitor, 3-(3, 4-dichlorophenyl)-1-1 dimethyl-urea (DCMU), caused *DIN2* (and other *DIN* genes) transcript to be detected even under illumination conditions (Fujiki *et al*, 2001). Hence a de-repression of *DIN2* transcript occurs when photosynthesis cannot. Taken together, these observations suggest that *DIN2* expression is likely to arise from a reduction in cellular sugar levels: in dark conditions, photosynthesis ceases and sugar

metabolism in initiated, causing elevated expression of dark inducible genes (Fujiki *et al*, 2001).

The signalling pathways involved in *DIN2* transcription have been examined in some detail. Sugar modulated expression of *DIN* genes is regulated by protein phosphorylation and dephosphorylation (Fujiki *et al*, 2000b). *DIN2* is known to be induced by *YLS4,* a senescence marker gene and is also upregulated in the early senescence mutant *hys1* (Fujiki *et al*, 2005). Changes in Ca^{2+} signalling are also thought to be important as calmodulin antagonists accelerate expression of *DIN2* in darkness but not light. Thus Ca^{2+}/ calmodulin signalling conveys a negative signal that suppresses the responses of late responsive *DIN* genes to prolonged darkness (Fujiki *et. al*, 2005). In conclusion, *DIN2* is thought to contribute to plant survival mechanisms during periods of sugar starvation through metabolism of β-glycoside conjugates within cellulose as alternate carbohydrate sources. Regulation of this process likely involves both transcriptional and calcium-dependent signalling mechanisms.

This chapter seeks to identify potential regulation of *DIN2* expression by changing R:FR ratio light conditions. In addition, this section examines the roles of phytochromes in R:FR ratio induced regulation of *DIN2* transcription. A possible gating of this response by the circadian clock is also studied. Through isolation and characterisation of *din2* mutants and over expressing lines, insight is gained into the possible physiological role of DIN2 during shade avoidance. Finally, the possibility that increases in cellular glucose levels induced by morphological changes required for shade avoidance is explored.

4.2. Results

4.2.1. The effect of low R:FR ratio on DIN2 transcript abundance

DIN2 expression was found by an Affymetrix oligoarray analysis, to respond to a 1 h low R:FR ratio light treatment. This analysis, which investigated expression profiles of 8200 Arabidopsis genes (Salter *et al*, 2003; for details see section 1.4.1.), showed that a pulse of low R:FR ratio light increased *DIN2* transcript levels 16.1 fold 1 h after dawn. These initial observations led to speculation that *DIN2* plays a role in the molecular shade avoidance pathway.

In order to confirm this finding, Columbia seedlings (section 2.1.3.) were grown in 12 h light/ 12 h dark high R:FR ratio conditions for 5 days. On the 6th day, seedlings were exposed to a 1 h low R:FR ratio treatment commencing 30 min after dawn. A further set of seedlings were exposed to a 1 h low R:FR ratio exposure 90 min before the onset of dusk. Control seedlings remained in high R:FR ratio and were not exposed to low R:FR ratio. Tissue was harvested after each treatment and Quantitative RT-PCR was carried out (Section 2.6.1.2., 2.6.2.6.). The results show that *DIN2* does indeed respond robustly to a 1 h low R:FR ratio treatment, both at dawn and dusk (Figure 4.2.1.). The relative difference in transcriptional response at these 2 time points will be discussed in relevance to the circadian clock in section 4.2.6. In contrast, in high R:FR ratio *DIN2* relative transcript remains equivalent at both dawn and dusk. This result confirmed that the dark induced *DIN2* gene is also low R:FR ratio light regulated.

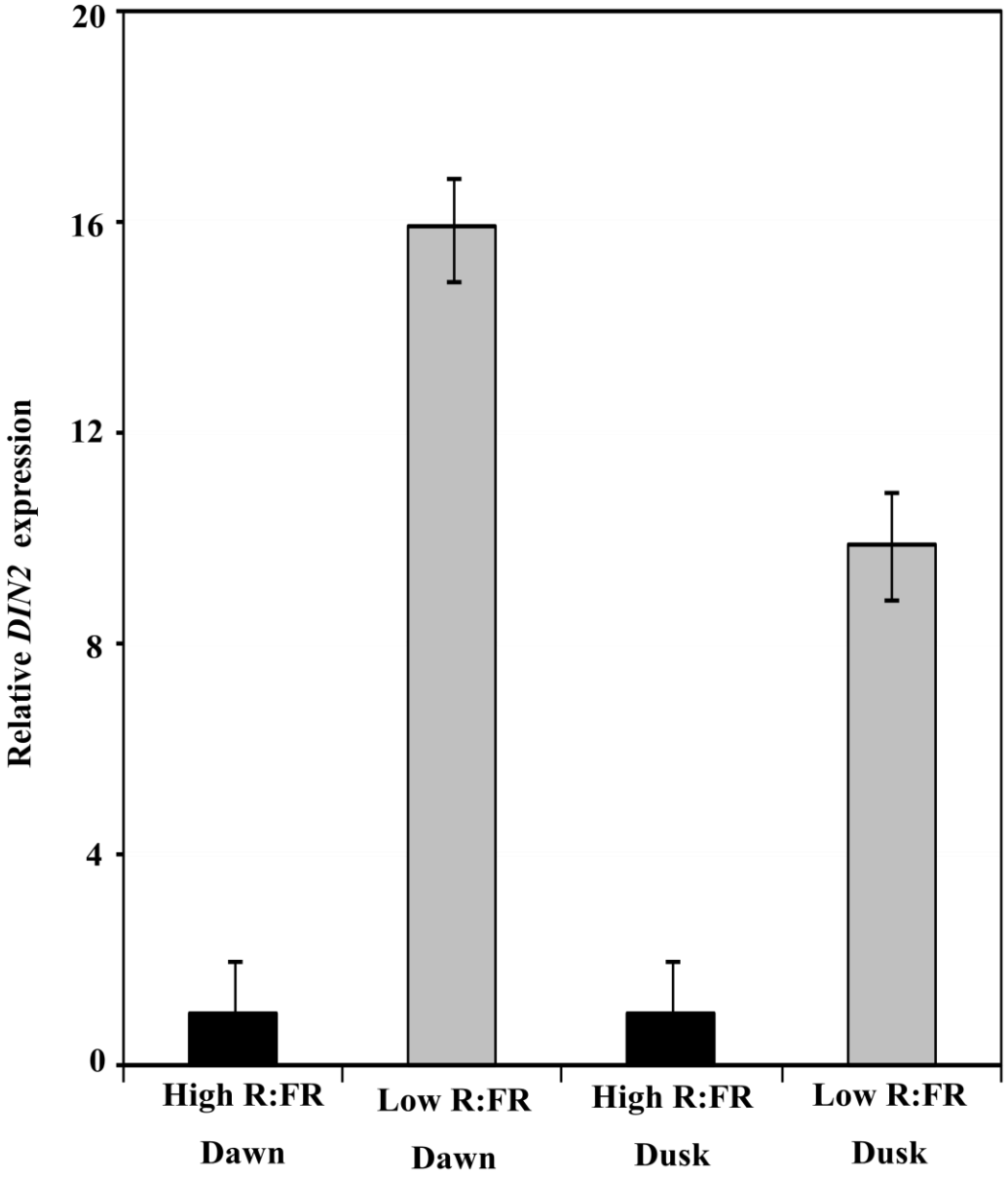


Figure 4.2.1. Analysis of *DIN2* transcript regulation in wild-type seedlings grown at 22°C in response to 1 h low R:FR (grey bars), compared with the control (black bars). The dusk treatment ended 30 min before lights went off. Transcript abundance of *DIN2* was measured using Quantitative RT-PCR. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalised to *ACTIN2*. Relative transcript is normalised to the high R:FR ratio grown control sample.

4.2.2. Kinetics of the DIN2 response to changes in R:FR ratio

As discussed previously, *DIN2* is known to be a late responsive dark inducible gene and transcript elevation has only previously been seen in leaves after 24 h of darkness. The work presented above demonstrates that by contrast, low R:FR ratio light causes rapid upregulation of *DIN2* transcription. Thus, the observation that *DIN2* mRNA increases within 1 h after a pulse of low R:FR ratio light suggests that this response occurs much more rapidly than that induced by dark exposure.

In order to determine more precisely the temporal nature of the *DIN2* transcriptional response to low R:FR ratio, Columbia seedlings were grown (as per the methods described in section 2.1.3.), in 12 h light/ 12 h dark high R:FR ratio light. On day 5, 30 min after the onset of dawn, seedlings were transferred to continuous low R:FR ratio, whilst controls remained in high R:FR ratio. At specific time points, Quantitative RT-PCR was conducted on samples taken from plants exposed to these conditions (Section 2.6.1.2., 2.6.2.6.). As shown in Figure 4.2.2.a, increased *DIN2* transcription is clearly observed after only 30 min low R:FR ratio light exposure, with peak levels occurring at around 4 h. Subsequently transcription begins to decline. A second, small elevation in transcript is also seen at 24 h, which suggests that the circadian oscillator also regulates *DIN2* transcription. In contrast, *DIN2* transcription is unaffected in high R:FR ratio light conditions. Thus, these findings demonstrate that elevation of *DIN2* transcription occurs very rapidly (within *ca*. 30 min) in low R:FR conditions, and gradually reaches peak levels (at *ca*. 4 h) before subsequently beginning to decline (at *ca*. 8 h). This contrasts with the dark-induced response which exhibits much slower kinetics.

Expression of many light-regulated genes, such as *PIL1*, is R:FR reversible. These genes are rapidly de-repressed by low R:FR ratio and are equally rapidly

repressed by high R:FR ratio conditions. To determine whether *DIN2* expression follows a similar pattern of R:FR ratio reversibility, experiments were performed whereby seedlings were grown in 12 h light/ 12 h dark high R:FR ratio (section 2.1.3.) until day 5 at which point they were exposed to 2 h continuous low R:FR ratio light and subsequently the seedlings were returned to high R:FR ratio light for a further 30 h. Figure 4.2.2.b demonstrates that when seedlings are returned to high R:FR ratio light conditions, a drop in *DIN2* relative transcript abundance can be observed after around 2 h. However, expression levels remained elevated when compared to seedlings that remained in continuous high R:FR ratio conditions.

The observed low R:FR ratio light induced modification of *DIN2* transcription suggests that the phytochrome signalling may underlie the rapid modulation of *DIN2* expression. The results also show that *DIN2* transcript falls very rapidly after transfer from low R:FR ratio to high R:FR ratio which is likely to be due to the photoconversion of phytochrome.

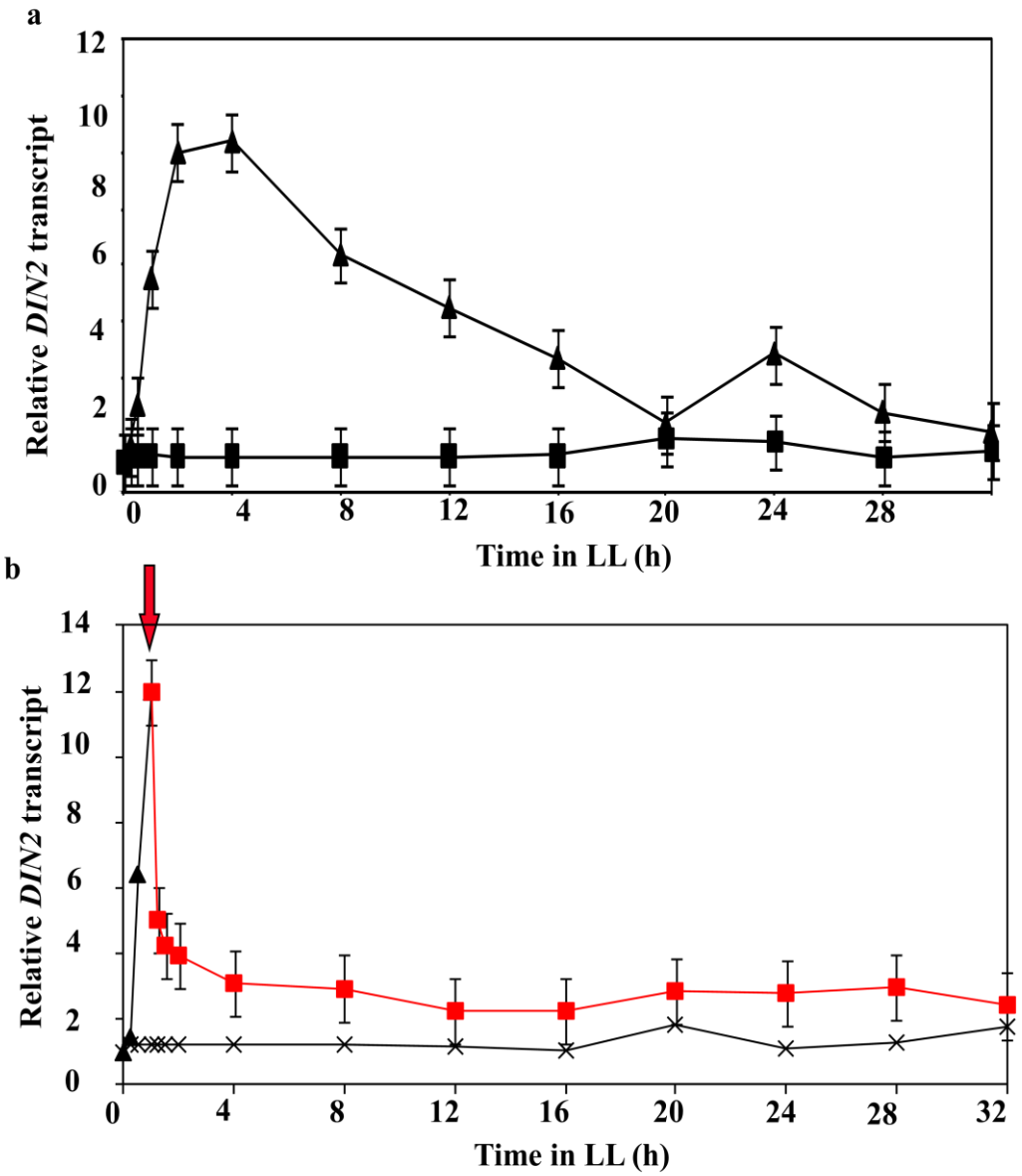


Figure 4.2.2. Quantitative RT-PCR of *DIN2* transcript in response to low R:FR ratio in wild-type seedlings. a. Rapid time course showing the increase in *DIN2* transcript abundance in seedlings transferred to continuous low R:FR ratio (▲) and the control seedlings which remained in high R:FR ratio (■). b. Time course of *DIN2* transcript in seedlings transferred to low R:FR ratio light treatment for 2 h (▲) and then the seedlings were transferred back to high R:FR ratio (red ■) for a further 30 h. The arrow indicates the time point at which the seedlings were moved back into continuous high R:FR ratio. Control seedlings remained in continuous high R:FR ratio (x). Each value is the mean of 3 separate Quantitative RT-PCR reactions normalised to *ACTIN2*. Relative transcript is normalised to the high R:FR ratio grown control sample.

4.2.3. Phytochrome regulation of *DIN2*

Previous studies have reported repression of *DIN2* transcription in white light exposed leaves (Fujiki *et al*, 2000). Nonetheless, the results presented in the previous section show that *DIN2* transcription is also controlled by low R:FR ratio light in an extremely rapid manner. As phytochromes are the photoreceptors that perceive changes in R:FR ratio light, it was important to determine which, if any, phytochromes regulate light induced changes in *DIN2* expression.

4.2.3.1. Phytochrome B regulation of DIN2

To address the aforementioned problem, phytochrome deficient mutants were studied. Wild-type and phytochrome mutant seedlings were grown in 12 h light/ 12 h dark high R:FR ratio for 5 days (section 2.1.3.). Tissue samples were taken 1 h after the onset of dawn on day 6 and Quantitative RT-PCR was subsequently performed (Section 2.6.1.2., 2.6.2.6.). Figure 4.2.3.1.a shows that phyB is the key repressor of *DIN2* transcription under high R:FR ratio conditions. This is illustrated by the finding that the *phyB* null has constitutively high *DIN2* transcript abundance. Interestingly *DIN2* transcript levels in *phyA* mutants are markedly reduced when compared to wild-type, suggesting that phyA de-represses *DIN2* under high R:FR ratio conditions. Single *phyD* and *phyE* mutants also have reduced relative *DIN2* transcript levels when compared to wild-type, suggesting that they too act to de-repress *DIN2* transcript in high R:FR ratio. Interestingly, analysis of *phyBD* double and *phyBDE* triple mutants revealed that relative *DIN2* transcript is slightly greater when compared with the single *phyB* mutant. Thus phy D and E may play a small additional role in *DIN2*

regulation. However, it seems that phyB is primarily involved in *DIN2* repression under high R:FR ratio conditions.

4.2.3.2. Phytochrome A regulation of DIN2

In low R:FR ratio conditions, phyB is primarily in its inactive Pr conformation and this is when shade avoidance genes are de-repressed, thereby permitting appropriate morphological changes in plant architecture. Phytochrome A plays an antagonistic role in the shade avoidance response: In low R:FR ratio conditions it acts to temper elongation growth in order to promote survival. Hence, genes which are de-repressed in low R:FR ratio conditions such as *PIL1* and *ENDO1* are often partially repressed by phyA. The clear result that phyB mediates repression of *DIN2* in high R:FR ratio conditions, suggested that phyA may act to repress *DIN2* transcript in low R:FR ratio light. To determine whether this was the case, mutants null for phyA and La-*er* were grown (see section 2.1.3.) in 12 h light/ 12 h dark light cycles for 5 days, the seedlings were then given 1 h low R:FR ratio commencing at the onset of subjective dawn. Tissue was subsequently harvested and subjected to Quantitative RT-PCR (2.6.1.2., 2.6.2.6.). The results, depicted in Figure 4.2.3.1.b show that phyA does indeed repress *DIN2* transcript in low R:FR ratio conditions, as in a *phyA* mutant there is an elevation of *DIN2* transcript when mutant plants are exposed to this light condition. The rapid increase in *DIN2* transcription upon transfer to low R:FR ratio light, a response likely mediated by reduction of phyB Pfr, is attenuated by the action of phyA.

Previous findings demonstrate that In high R:FR ratio light conditions, phyB is mostly in its biologically active conformation, and acts to repress *DIN2* transcription. This repression of *DIN2* transcript in white light conditions has been documented by Fujiki *et al*, (2001). However, the results presented in this section have shown that in

low R:FR ratio light conditions, a clear de-repression occurs, which is likely a result of phyB being principally in its biologically inactive state. These results suggest that *DIN2* is controlled in a far more complex manner than has previously been suggested.

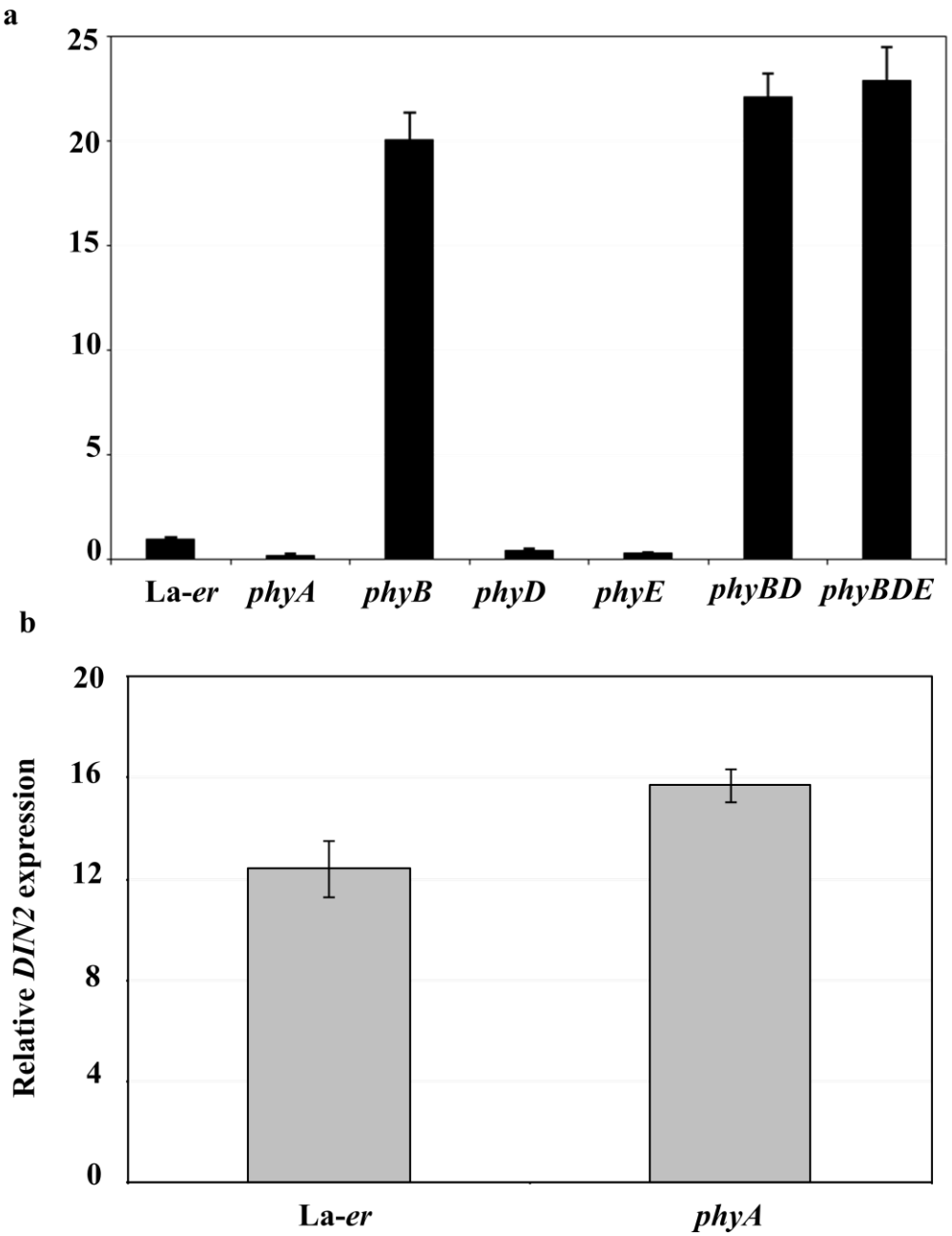


Figure 4.2.3.1. Phytochrome-regulation of *DIN2* transcript abundance. a. Transcript abundance of *DIN2* was measured in 5 day old phytochrome mutants grown in 12 h light/ 12 h dark cycles at 22°C using Quantitative RT-PCR. Relative transcript abundance is normalized to the wild-type control (La *er*). b. Relative expression of *DIN2* transcript in response to 1 h low R:FR ratio light treatment commencing at subjective dawn (grey bars). Relative transcript expression is normalised to *DIN2* levels in high R:FR ratio grown la-*er* grown plants. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalized to *ACTIN2*.

4.2.4. The role of dark induction and sugar starvation in *DIN2* regulation

4.2.4.1. The effect of dark and sucrose on DIN2

As discussed previously, Fujiki and colleagues (2001) showed long periods of darkness induced *DIN2* transcript accumulation in leaves, a phenomenon which could be abolished by exposure to high concentrations of sugar (Fujiki *et al*, 2001). From these results Fujiki *et al* concluded that *DIN2* expression was induced by a reduction in cellular sugar levels. To confirm this finding and examine in detail the kinetics of this response, seedlings were grown (as per section 2.1.3.) in continuous high R:FR ratio conditions on Lehle plates with or without sucrose (section 2.1.1.) for 5 days. On day 6 they were either moved to continuous darkness or remained in continuous high R:FR ratio light. Samples were taken at various time points and Quantitative RT-PCR subsequently carried out (2.6.1.2., 2.6.2.6.). The results (illustrated in figure 4.2.4.1.) demonstrate that whilst dark-exposed seedlings grown in the absence of sucrose exhibit elevated *DIN2* transcription after 20 h of darkness, those grown in 3% sucrose do not show a marked change in *DIN2* transcript. Thus sucrose acts to repress *DIN2* transcript in the dark. However, the results also show slightly elevated steady-state *DIN2* transcript abundance in seedlings grown in darkness with sucrose when compared to those grown in continuous light without sucrose. This finding suggests that sucrose can only partially repress *DIN2* transcript in darkness. Thus, two separate pathways; a light regulatory pathway and sugar starvation pathway; could regulate *DIN2* expression.

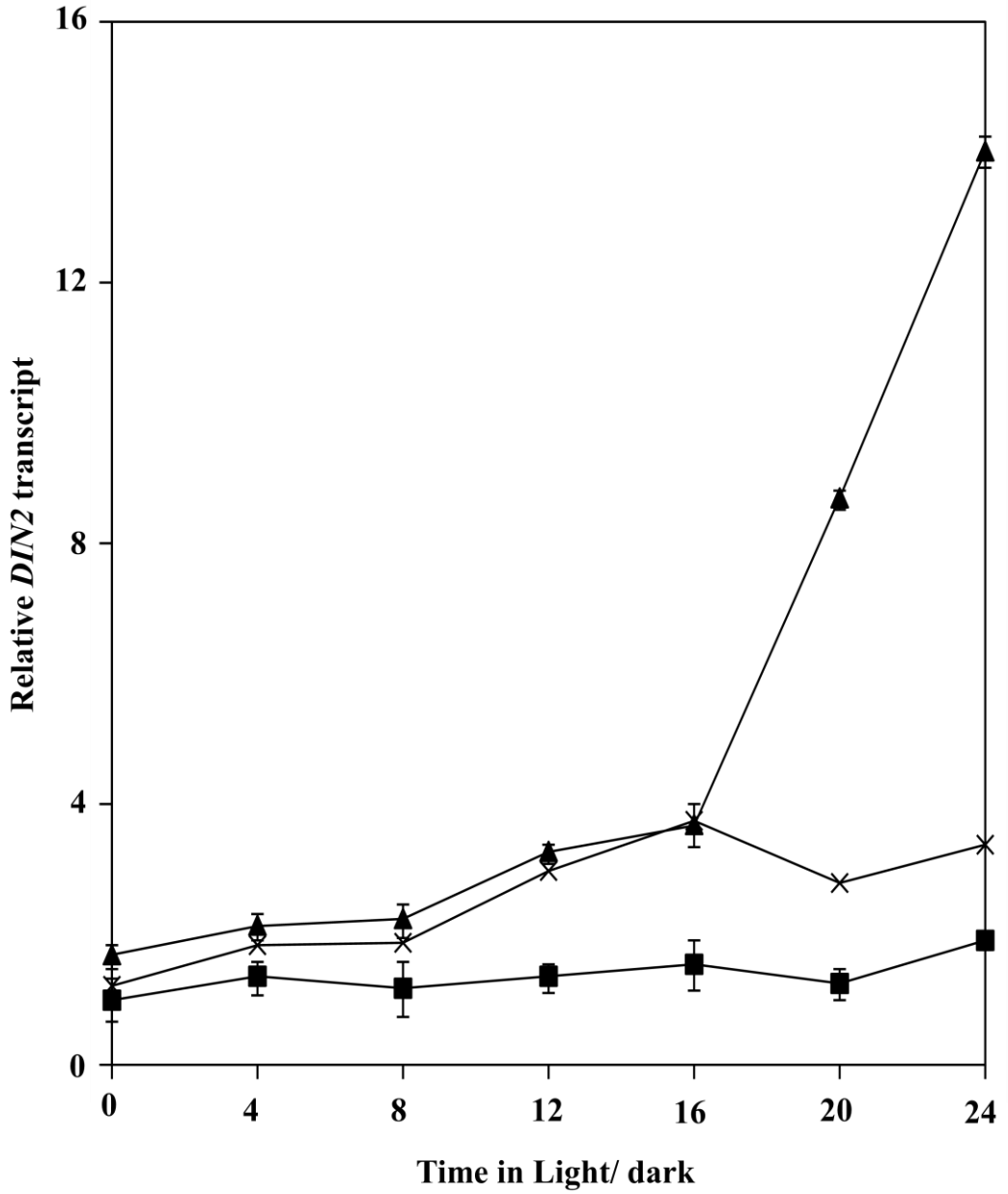


Figure 4.2.4.1. Time course analysis of *DIN2* transcript regulation in seedlings in high R:FR ratio without sucrose (■), in darkness with 3% sucrose (**x**) and in darkness without sucrose (▲) at 22°C. Transcript abundance of *DIN2* was measured using Quantitative RT-PCR. Each value is the mean of 3 separate reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to the high R:FR ratio grown sample at time 0. Variation between individual reactions is less than 2%.

4.2.4.2. The effect of sucrose and low R:FR ratio on DIN2 transcript

The results show that *DIN2* transcript is clearly induced after 20 h of darkness and this effect can be partially repressed by exogenous sugar exposure. In the previous section, *DIN2* transcript formation has been shown to be repressed in high R:FR ratio light conditions (presumably through phyB mediated signalling) whilst it is de-repressed in low R:FR ratio light conditions. One possible explanation for the observed change in *DIN2* transcript is that altering R:FR ratio light conditions causes a depletion of cellular sugar levels which concomitantly triggers an increase in *DIN2* transcript. This raises the possibility that both sugar and light responsive pathways are interlinked. In order to investigate this prospect, seedlings were grown on media with or without 3% sucrose (section 2.1.1.). Seedlings were germinated (section 2.1.3.) and grown in 12 h light/ 12 h dark high R:FR ratio conditions for 5 days and on day 6 at dawn they were given a transient 1 h low R:FR ratio response. The controls remained in high R:FR ratio. Samples were then taken and Quantitative RT-PCR conducted (2.6.1.2., 2.6.2.6.). The results (figure 4.2.4.2.) show that seedlings grown on sucrose-free media, exhibit a robust de-repression of *DIN2* transcript after 1 h of low R:FR ratio light exposure. In contrast, whilst seedlings grown on a sucrose containing media still show a response to low R:FR ratio light, it is markedly attenuated. This indicates that sucrose does act to repress *DIN2* in low R:FR ratio conditions but it alone is not sufficient to completely abrogate transcriptional up-regulation in this light condition. The observation that supplementary sucrose reduces, but does not eliminate completely, the response of *DIN2* to low R:FR ratio, suggests that *DIN2* is not simply regulated by either sucrose levels or changes in R:FR ratio, but rather it is subjected to regulation by both stimuli.

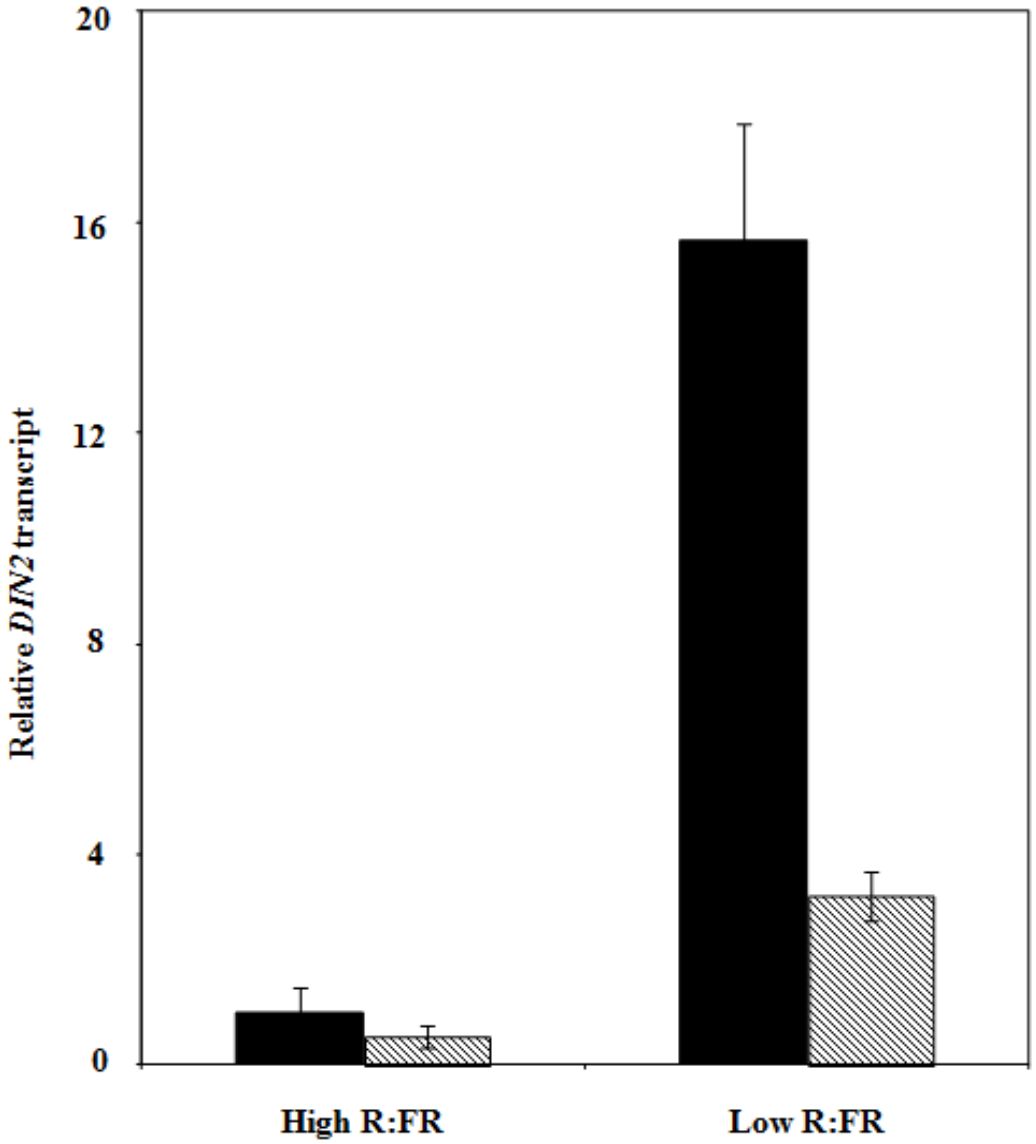


Figure 4.2.4.2. Regulation of *DIN2* transcript abundance. Transcript abundance of *DIN2* in response to a 1 h low R:FR ratio pulse measured using Quantitative RT-PCR. Columbia seedlings were grown for 6 days in 12 h light/ 12 h dark cycles at 22°C on media (black bars) or media containing 3% sucrose (grey bars). Each value is the mean of 3 separate Quantitative RT-PCR reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to the wild-type control high R:FR ratio (grown on sucrose free media).

4.2.5. Transcriptional regulators of *DIN2*

It is well established that light-induced conversion of the phytochrome molecule to its inactive Pr form, resulting in de-repression of transcriptional regulators that can then trigger an intracellular signalling process that culminates, within minutes, in the altered expression of target genes. It has now been demonstrated that member of the phytochrome family, in particular – phyB regulates *DIN2* transcription. Despite this, little is currently known of the transcriptional regulators acting downstream of phyB that are involved in this process.

4.2.5.1. Phytochrome interacting factor 3 regulation of DIN2

A known light responsive bHLH transcription factor is PIF3: A shift in phyB equilibrium to the Pfr conformation leads to an alteration in PIF3 protein levels and by corollary, this protein could potentially act as a molecular component of the *DIN2* transcriptional regulation machinery. To test as to whether *PIF3* does indeed regulate *DIN2* transcription, an experiment was set up using both *pif3* mutants and *PIF3* over-expressers. Seeds were germinated and grown (section 2.1.3.) in 12 h light/ 12 h dark high R:FR ratio for 5 days. On day 6, at subjective dawn the seedlings were subjected to 1 h high R:FR ratio or 1 h low R:FR ratio. Tissue samples of the seedlings were then taken and processed for Quantitative RT-PCR (2.6.1.2., 2.6.2.6.). The results (figure 4.2.5.1.) show that in wild-type seedlings *DIN2* transcription is elevated after 1 h low R:FR ratio light exposure. Interestingly in *pif3* mutants, *DIN2* relative transcript is slightly elevated after 1 h low R:FR ratio when compared to wild-type. More obviously, the over-expression of *PIF3* results in a significant decrease in *DIN2* transcript when compared to wild-type. It is also notable that in high R:FR ratio light

the Columbia *pif3* mutant has 2-fold greater levels of *DIN2* relative transcript when compared to wild-type. This is the case when comparing Ws *pif3* mutant in with Ws. This suggests that in high R:FR ratio conditions, PIF3 acts to repress *DIN2* transcript. In conclusion, the light regulated transcription factor PIF3 negatively regulates *DIN2* in response to low R:FR ratio conditions and may also negatively regulate *DIN2* in high R:FR ratio conditions.

4.2.5.2. Phytochrome interacting factors 4 and 5 regulation of DIN2

PIF4 and *PIF5* are two close homologues in the PIF protein family of PIF proteins. Transgenic plants that over-express either gene exhibit a constitutive shade avoidance phenotype. Despite this, *pif4*, *pif5* and *pif4pif5* mutants still respond to a reduction in R:FR ratio. These phyB binding proteins are known to regulate the expression of low R:FR ratio induced genes such as *PIL1*, *HFR1* and *ATHB-2* (Allen, pers com, 2006). PIF4 and PIF5 over-expressing lines show constitutively high levels of *PIL1*, *HFR1* and *ATHB-2*. Interestingly, in response to low R:FR ratio *pif4*, *pif5* mutants, and in particular the *pif4pif5* double mutant, has reduced *PIL1*, *HFR1* and *ATHB-2* transcript abundance.

Both PIF4 and PIF5 are rapidly degraded when exposed to high R:FR light, and accumulate quickly in response to low R:FR ratio light. Thus it is likely that these proteins have R:FR reversible actions in the molecular pathway. Given this, it is possible that *PIF4* and *PIF5* underpin R:FR ratio mediated changes in *DIN2* transcript turnover. To test this hypothesis *DIN2* transcript was measured in the relevant mutants, full length PIF5 over-expresser, ΔN PIF5 or PIF4 over-expressers. The ΔN PIF4 and PIF5 over-expressers are constitutively active lines lacking the N-terminus APB binding domains required for phyB-mediated degradation. Thus, in

these lines, PIF4 and PIF5 are not subject to regulation by phyB. The net effect is that expression of these proteins is higher than wild type plants.

Full length PIF5 over-expresser, ΔN PIF5 or PIF4 over-expressers germinated, and grown (section.2.1.3.) for 5 days in 12 h light/ 12 h dark high R:FR ratio light. On day 6 the seedlings were given a 1 h pulse of low R:FR ratio light commencing at subjective dawn whilst, controls remained in high R:FR ratio conditions. Samples were then taken and Quantitative RT-PCR carried out (2.6.1.2., 2.6.2.6.). Figure 4.2.5.2. shows that in both *pif4* and *pif5* mutants, low R:FR ratio mediated changes in *DIN2* expression are similar to wild-type. In contrast, *pif4pif5* double mutant exhibited attenuated *DIN2* expression in response to low R:FR ratio light. Over-expression of PIF5 and ΔN PIF4 resulted in higher endogenous *DIN2* transcript and a reduced response to low R:FR ratio signals. This result indicates that *PIF4* and *PIF5* act to positively regulate *DIN2* in the molecular shade avoidance pathway. Interestingly, in high R:FR ratio conditions the single mutants *pif4*; and to a greater extent *pif5*; show a decrease in relative *DIN2* transcript. This indicates that in high R:FR ratio conditions, *PIF4* and *PIF5* again act to positively regulate *DIN2*.

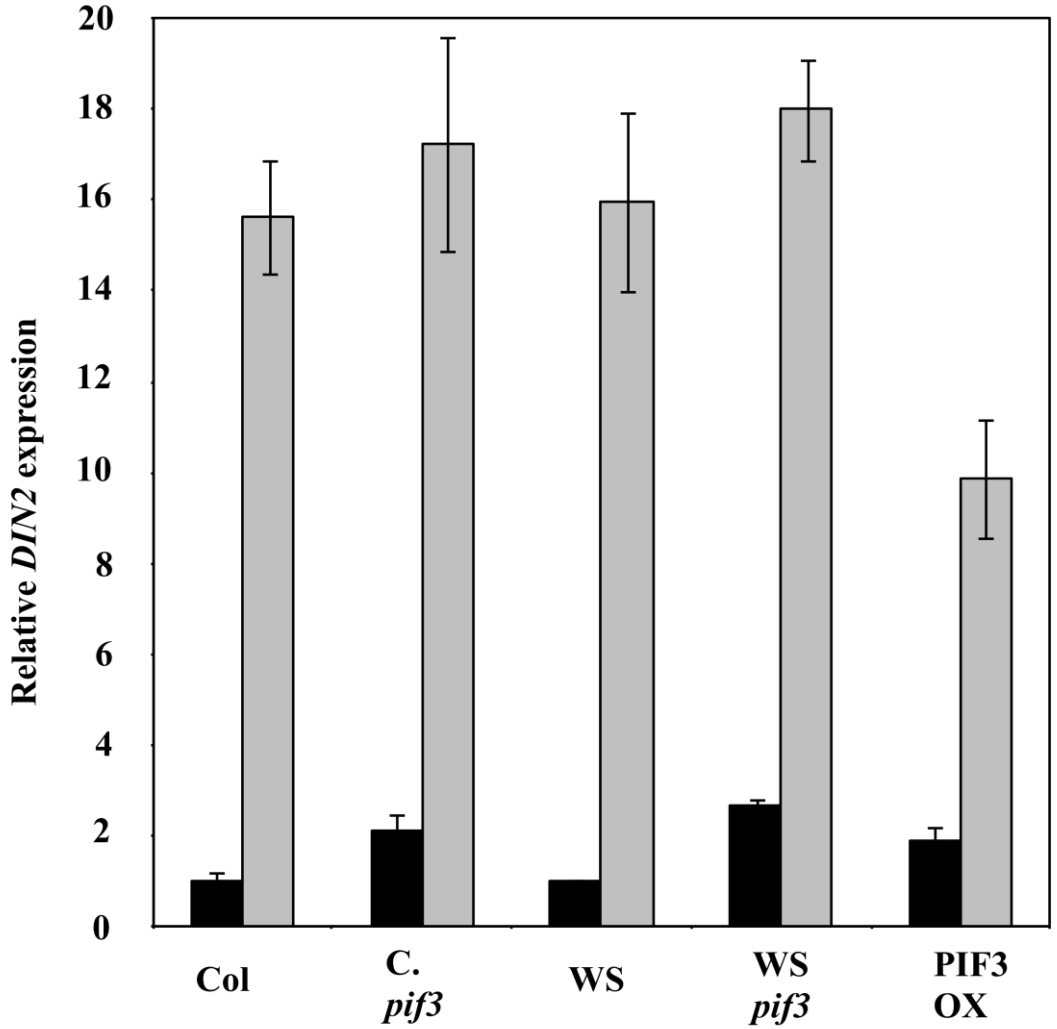


Figure 4.2.5.1. Transcript abundance of *DIN2* was measured using Quantitative RT-PCR in 5 day-old *pif3* mutants and over-expressers in response to 1 h low R:FR ratio treatment at subjective dawn (gray bars). Controls remained in high R:FR ratio (black bars). All seedlings were grown in 12 h light /12 h dark cycles at 22°C. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalised to *ACTIN2*. Relative transcript abundance is normalised to the relevant high R:FR ratio grown wild-type sample.

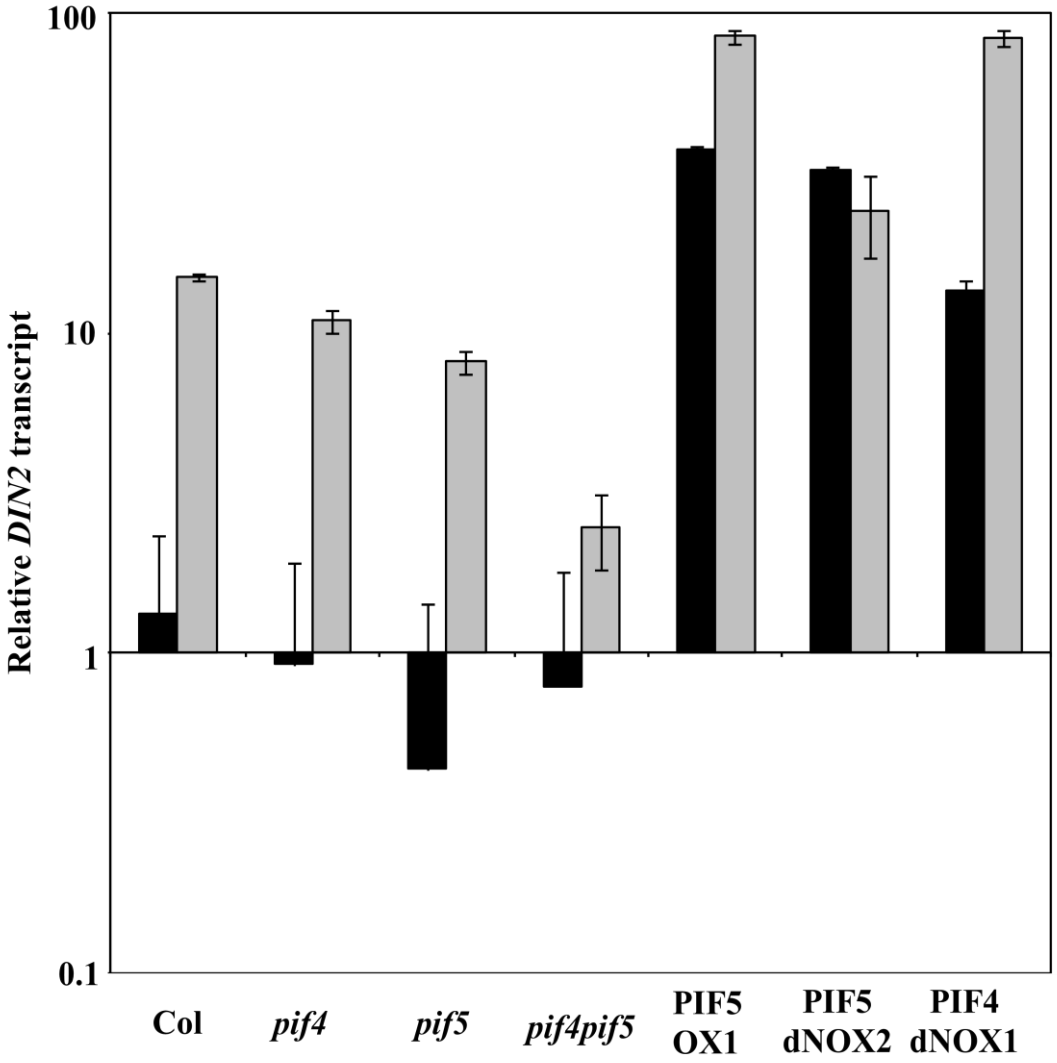


Figure 4.2.5.2. Transcript abundance of *DIN2* was measured using Quantitative RT-PCR in 5 day-old *pif4 and pif5* mutants and over-expressers in response to 1 h low R:FR ratio treatment at subjective dawn (gray bars). Controls remained in high R:FR ratio (black bars). All seedlings were grown in 12 h light /12 h dark cycles at 22°C. Each value is the mean of 3 separate quantitative RT-PCR reactions normalised to *ACTIN2*. Relative transcript abundance is normalised to the relevant high R:FR ratio grown wild-type sample at dawn.

4.2.6. Circadian regulation of *DIN2*

DIN2 was initially identified by oligoarray analysis to be responsive to a 1 h pulse of low R:FR ratio light. The gene was found to show a 16.1 fold-increase in transcript 1 h after subjective dawn and again 24 h later. This result suggests that *DIN2* transcript levels may oscillate with a periodicity of 24 h and, like many other low R:FR ratio regulated genes, may be controlled by the circadian clock. Subsequently it was found that *DIN2* transcriptional responses to low R:FR ratio light pulses vary according to the time of day (Figure 4.2.1.). A 1 h transient low R:FR ratio pulse appears to cause greater de-repression of *DIN2* transcript at subjective dawn than at subjective dusk. However, the precise temporal nature of this response had not been determined.

In order to examine the effect of low R:FR ratio on *DIN2* transcription at various time points throughout the day, wild-type plants were germinated and grown (section 2.1.3. and section 2.2.4.) for 21 days in 12 h light/ 12 h dark high R:FR ratio cycles. Commencing at dawn on day 22, plants were moved into continuous high R:FR ratio conditions and given a 1 h pulse of low R:FR ratio at various time points. Samples were taken after each treatment and Quantitative RT-PCR was carried out (2.6.1.2., 2.6.2.6.). The results show that expression of *DIN2* transcript in low R:FR ratio conditions is cyclic (figure 4.2.6.). Whilst a 1 h low R:FR ratio pulse causes de-repression of *DIN2* transcript at all time points, maximum de-repression of *DIN2* transcription occurs at subjective dawn. Interestingly, data so far has shown that most known low R:FR ratio regulated genes controlled by the circadian clock show a peak at the beginning of the subjective day. No robust circadian rhythm in *DIN2* transcript is seen in high R:FR ratio conditions (figure 4.2.6).

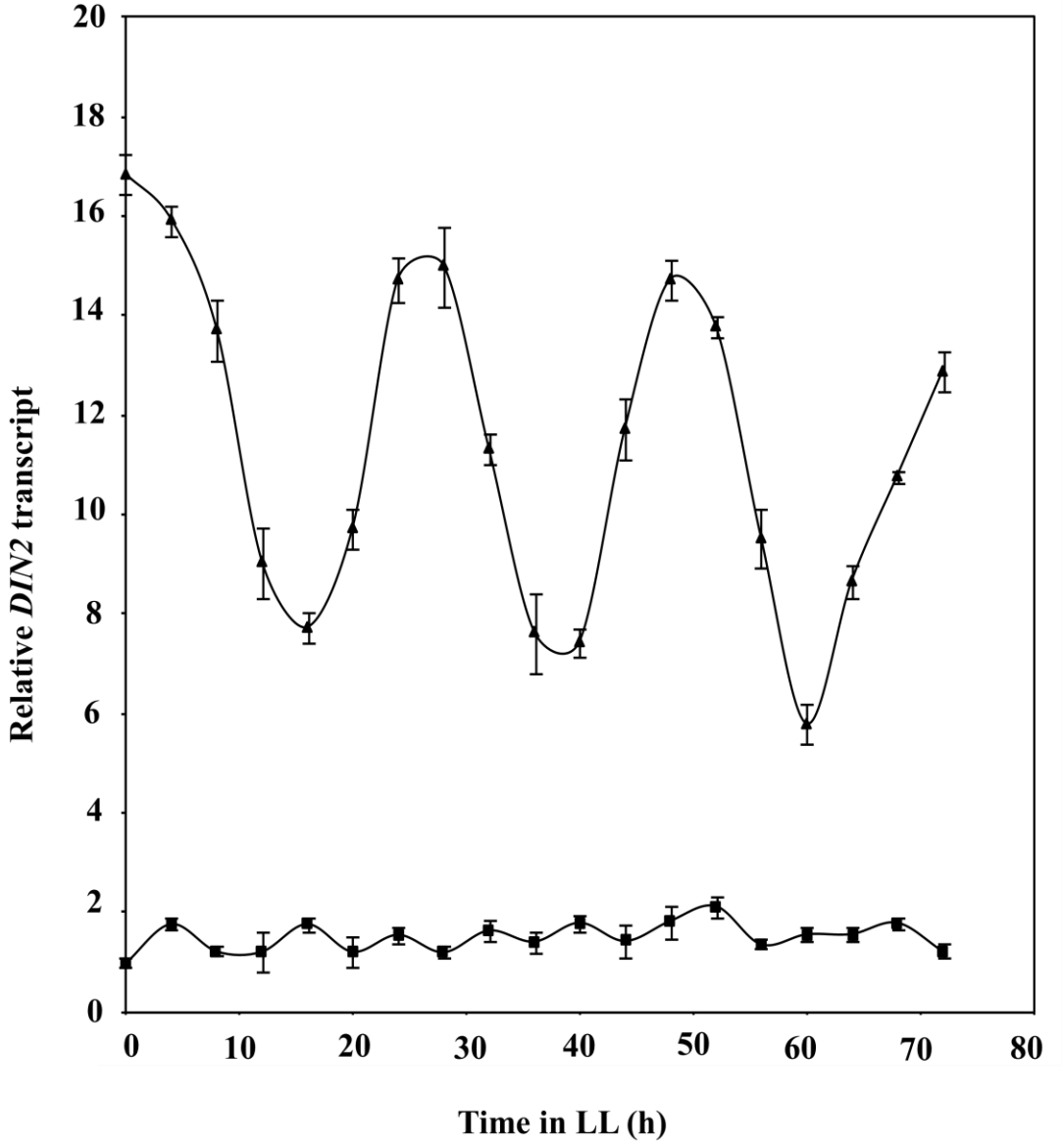


Figure 4.2.6. Circadian analysis of *DIN2* transcript regulation by low R:FR ratio (▲) at 22°C in wild-type plants. Controls remained in high R:FR ratio (■). Plants were grown in 12 h light/ 12 h dark for 21 days, on day 22 they received the various treatments. Transcript abundance of *DIN2* was measured using Quantitative RT-PCR. Each value is the mean of 3 separate reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to the high R:FR ratio grown sample at time 0.

4.2.7. R:FR ratio regulation of *DIN6* and *DIN9*

There are a large number of other dark induced, sugar repressed genes which can be classed as either early or late responsive to dark conditions. Of these, *DIN2* is a late responsive dark induced gene. So far *DIN2* is the only dark induced gene known to also be affected by low R:FR ratio light. However, it is possible that other genes within this family respond in a similar fashion. To gain insight as to whether this could be the case two other *DIN* members were analysed; *DIN6* and *DIN9*. *DIN6* is known to be early dark-inducible gene (transcript elevation can be detected after only 3 h of darkness) whilst *DIN9* is a late dark-inducible gene (transcript elevation is detectable after 48 h of darkness (Fujiki *et al*, 2000)).

Wild-type seedlings were germinated (section 2.1.3.) and grown for 5 days in 12 h light/ 12 h dark high R:FR ratio conditions. On day 6 the seedlings were given a 1 h pulse of low R:FR ratio commencing at subjective dawn, samples were then harvested for Quantitative RT-PCR (2.6.1.2., 2.6.2.6.). The results, depicted in figure 4.2.7. indicate that transcript levels of *DIN6* and *DIN9* are unaffected by either high or low R:FR ratio light. Therefore, it appears that for these two light repressed genes at least, changes in R:FR ratio do not affect transcription. Of course, it is possible that other *DIN* genes are regulated in the same fashion as *DIN2*. Future experimental analysis will help determine whether this is indeed the case.

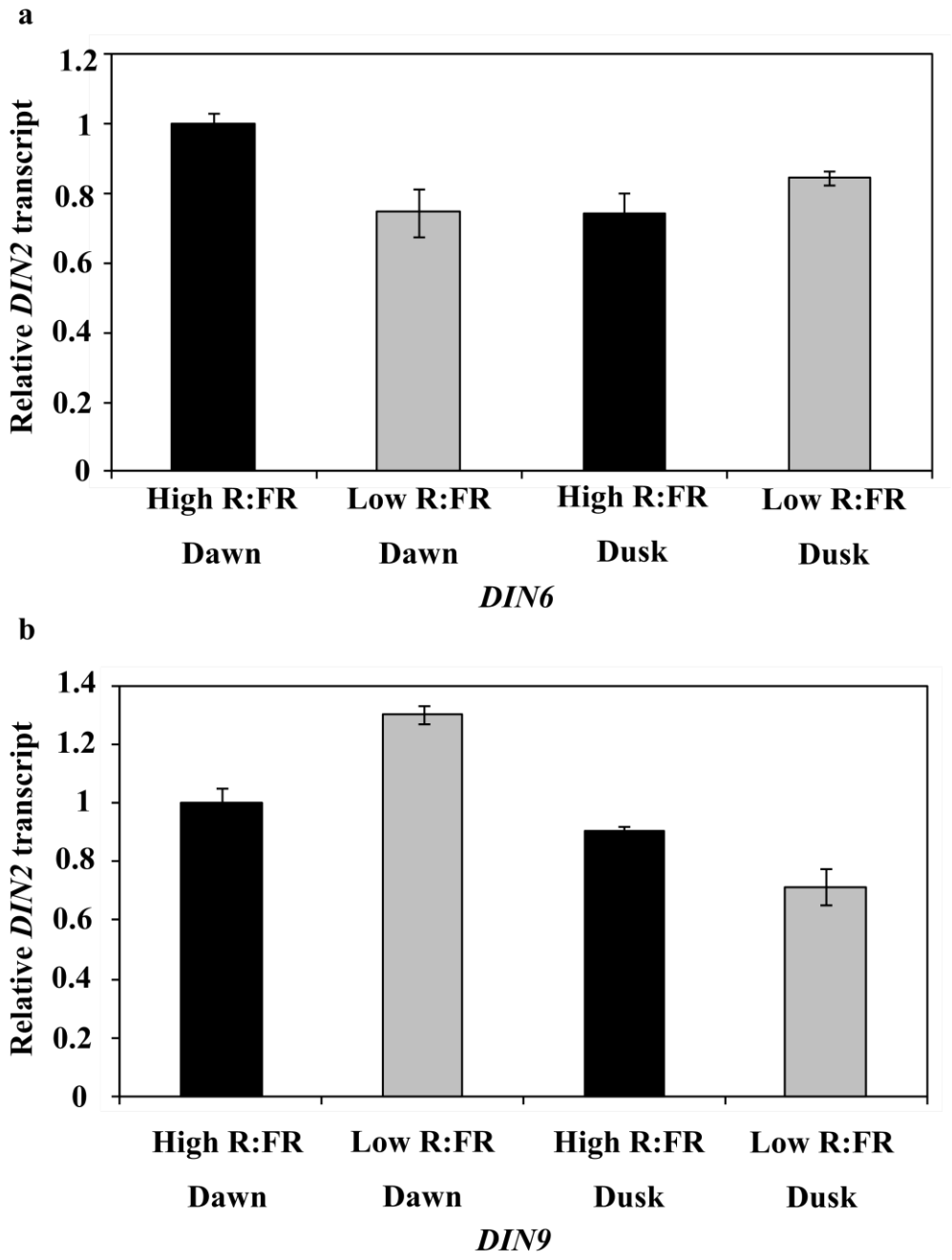


Figure 4.2.7. Quantitative RT-PCR analysis of *DIN* transcript levels following a 1 h treatment of low R:FR ratio at dawn. Controls remained in high R:FR ratio. The wild-type seedlings were 5 days old grown in 12 h light/ 12 h dark at 22°C. a. Relative *DIN6* transcript. b. Relative *DIN9* transcript. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to wild-type high R:FR ratio sample.

4.2.8. Determining a biological role for *DIN2*

The morphology of a plant is determined by a number of environmental cues, one of the most important of which is light. The aforementioned sections clearly show a light-mediated regulation of *DIN2* expression. Given this, it is possible plants lacking functional *DIN2,* show altered morphology in response to light quality changes. However, it is worth noting that although light is a key environmental stimulus, it is unlikely to act in isolation. Other environmental cues are likely to act in concert with light to bring about changes in plant growth. It is therefore plausible that crosstalk occurs between a number of environmentally-modulated signalling pathways to bring about changes in development.

The role DIN2 plays in the shade avoidance phenotype has not yet been elucidated. The isolation and physiological study of both *din2* mutants and over-expressing lines will facilitate the determination of a biological role for this gene. The following sections detail how over-expression or loss of *din2* function affects physiological shade avoidance responses.

4.2.8.1. T-DNA insertion lines in DIN2

To determine the putative role of DIN2 in shade avoidance, two transgenic lines null in *DIN2* were obtained. These lines (*din2-1* and *din2-2*) were homozygous mutants with a single T-DNA insertion in an independent location on the gene. This was determined by the design of primers against the T-DNA insert sequence, enabling these regions to be amplified by PCR (see section 2.5.2.4.) and subsequently sequenced (section 2.5.2.7.). Mapping of the exact location of each insert revealed that the transgene was inserted into discrete regions of *din2-1* and *din2-2* (figure

4.2.7.1.a). RT-PCR also revealed that both mutants failed to synthesise detectable levels of *DIN2* transcript (figure 4.2.7.1.b), suggesting that they were indeed loss of function lines.

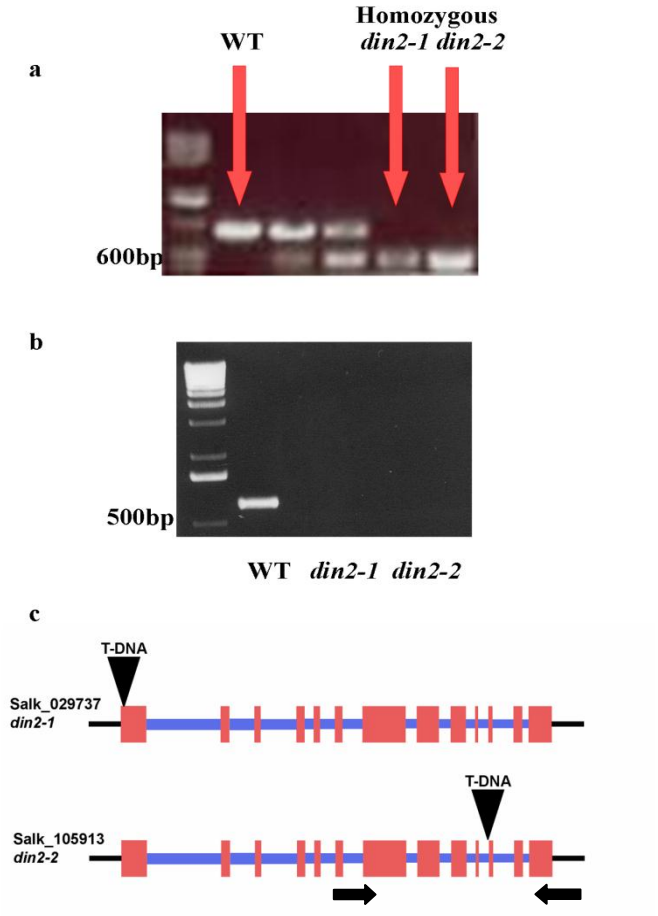


Figure 4.2.8.1. a. Genomic primers were designed and RT-PCR was carried out identifying the presence of the T-DNA insert in *din2-1* and *din2-2* lines. b. RT-PCR of *DIN2* transcript in wild-type, *din2-1* and *din2-2*. No *DIN2* transcript detected in din2-*1* and *din2-2* mutants. c. The position of the T-DNA inserts in the *din2* mutants. Knockout mutants were obtained from the Nottingham Arabidopsis stock centre (*din2-1* = SALK_029737 and *din2-2* SALK_105913).

c. The forward and reverse arrows represent the forward and reverse primers used for RT-PCR verification.

4.2.8.2. Over-expressing DIN2

DIN2 over-expressing lines (DIN2 OX) were constructed in the Columbia ecotype by sub-cloning the *DIN2* cDNA into the plant transformation vector pROK2 under the control of the CaMV 35S promoter. The Plant transformation was carried out and a transformed line was then selected using the resistance marker Kanamycin 40 μg ml^{-1}. For more detail see section 2.1.4. Quantitative RT-QPCR detected a clear over expression of transcript when compared to wild-type (Figure 4.2.8.2.).

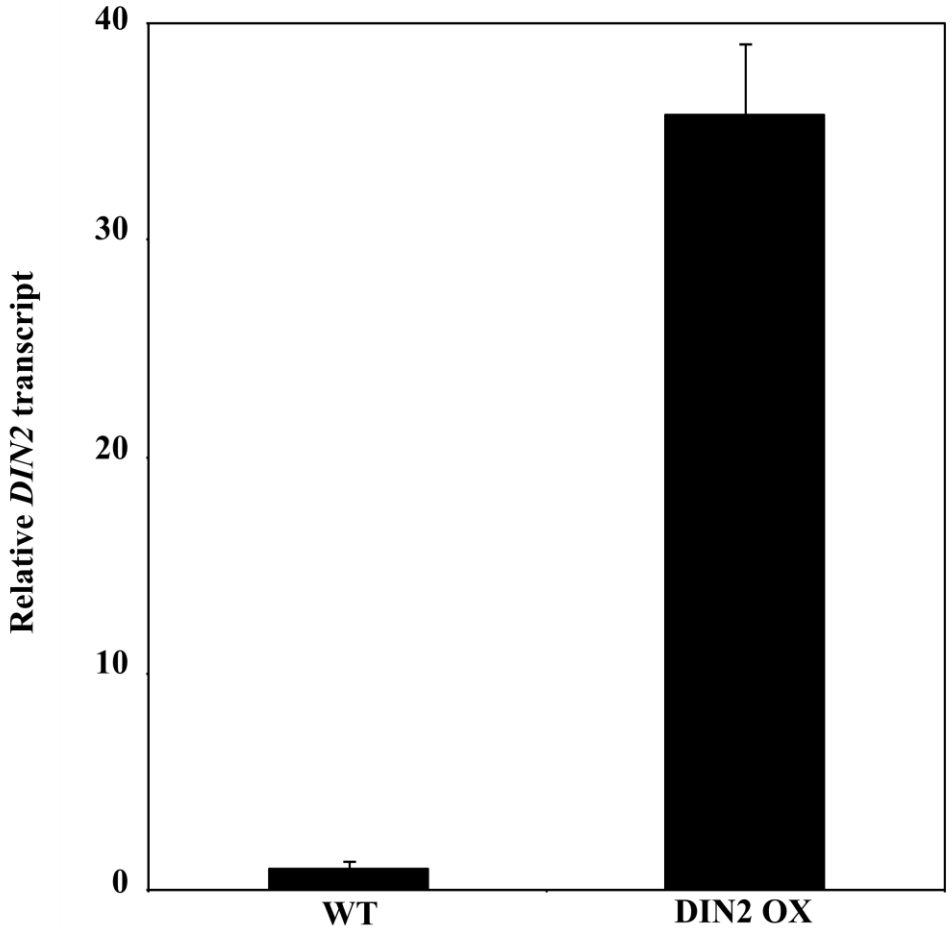


Figure 4.2.8.2. Analysis of *DIN2* transcript in the 35S::DIN2 over expressing construct in low R:FR ratio at 22°C. Transcript abundance of *DIN2* was measured using quantitative RT-PCR. Each value is the mean of 3 separate reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to the high R:FR ratio grown sample at time 0.

4.2.8.3. The effect of low R:FR ratio on hypocotyl elongation in din2 mutants and over-expressers

The enhancement of hypocotyl elongation in response to low R:FR ratio is one of the hallmarks of shade avoidance behaviour. It is known that a single, 2 h transient low R:FR ratio pulse is sufficient to induce a significant promotion of hypocotyl growth in seedlings, whereas a similar treatment at subjective dawn leads to an inhibition of hypocotyl elongation.

In order to test whether *DIN2* is necessary for this circadian regulated elongation response an experiment was set up whereby seedlings were grown (section 2.1.3.) for 5 days in 12 h light/ 12 h dark high R:FR ratio. On day 6 seedlings were given a 2 h pulse of low R:FR ratio which ended 30 min before the onset of dusk, the controls remained in high R:FR ratio (section 2.2.2.). The seedlings were then measured 24 h later using ImageJ (see section 2.2.6.). Figure 4.2.8.3. shows that low R:FR ratio responses in the mutant seedlings resemble that of wild-type: a promotion of approximately 26% growth is seen at dusk. It is therefore likely that *DIN2* is not an essential molecular component of the hypocotyl elongation response to low R:FR light.

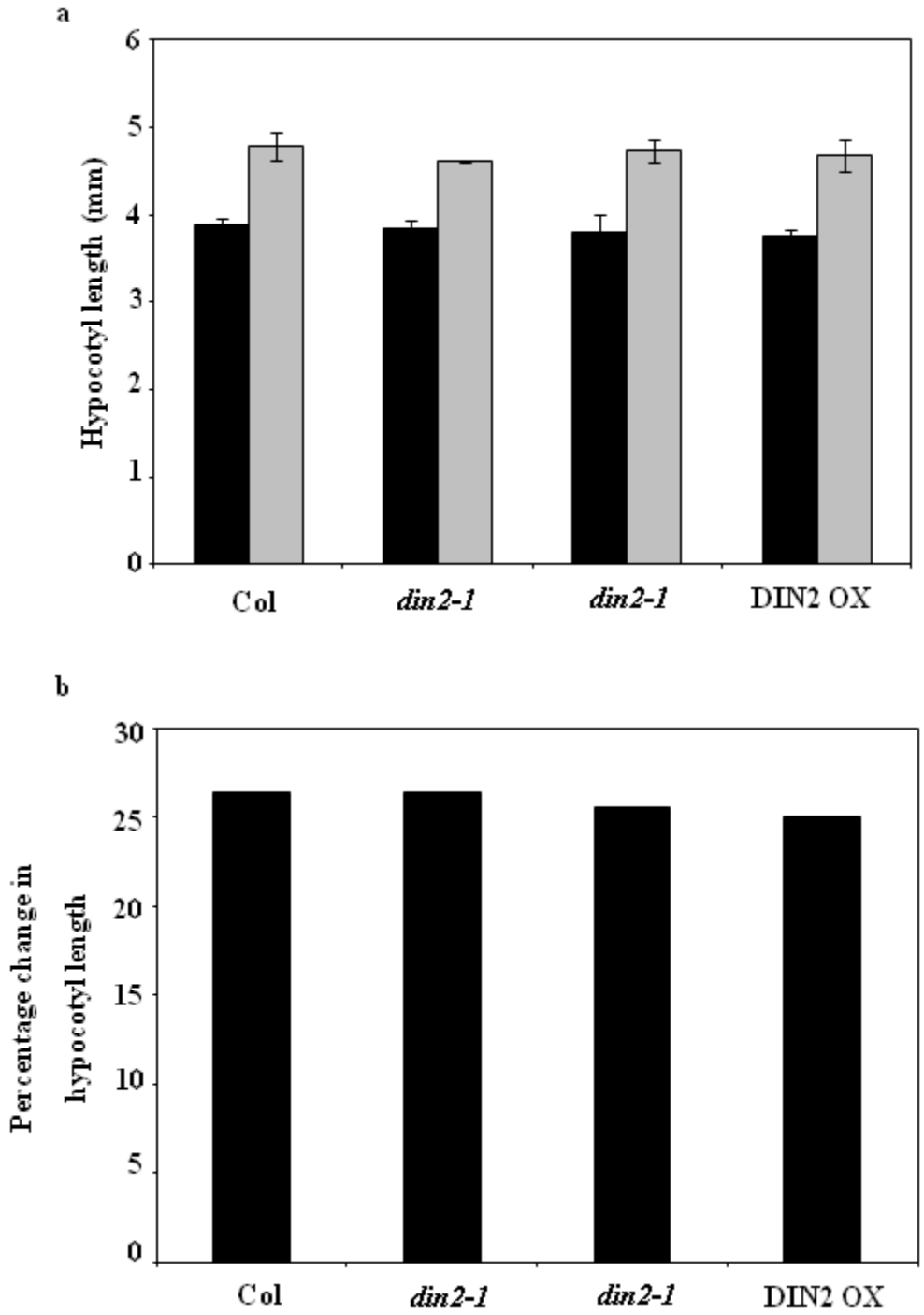


Figure 4.2.8.3. a. Hypocotyl lengths of the relevant wild-types, *din2* mutants and DIN2 OX seedlings, grown for 5 days in 12 h light/ 12 h dark cycles at 22°C and treated with single 2 h reduction in R:FR ratio ending 30 min (grey bars) before the end of the light period. Controls remained in high R:FR ratio (black bars). Hypocotyls were measured 24 h after the end of the treatment. Error bars represent SE. b. Shows percentage change in hypocotyl lengths. A sample size of 15 was used for each genotype.

4.2.8.4. The effect of reduced R:FR ratio on petiole elongation in din2 mutants and over-expressers

When plants are subjected to a reduction in R:FR ratio, a reallocation of resources results in stem and petiole elongation at the expense of leaf and storage organ development. It is known that this response is controlled at least in part by *PHYB*. In plants lacking functional *PHYB*, low R:FR ratio petiole elongation is severely attenuated. Previous results have shown that *DIN2* is regulated by phyB and therefore it is possible that petioles of *din2-1, din2-2* and DIN2 over-expressers could respond abnormally to a change in R:FR ratio.

To determine if this was the case, wild-type, *din2* mutants and DIN2 over-expressers were germinated (see section 2.1.3.) and grown for 14 days in 12 h/ light 12 h dark high R:FR ratio conditions and then they were moved to low R:FR ratio light dark cycles for a further 7 days (section 2.2.4.). Controls remained in high R:FR ratio light dark cycles. Petioles from leaf 5 were measured at the end of the 7 day treatment (section 2.2.7.). The results (figure 4.2.8.4.) show that the petiole response of *din2-1, din2-2* and DIN2 over-expressers are comparable to that of wild-type. As such, it appears that DIN2 is not essential for the petiole elongation response to low R:FR conditions.

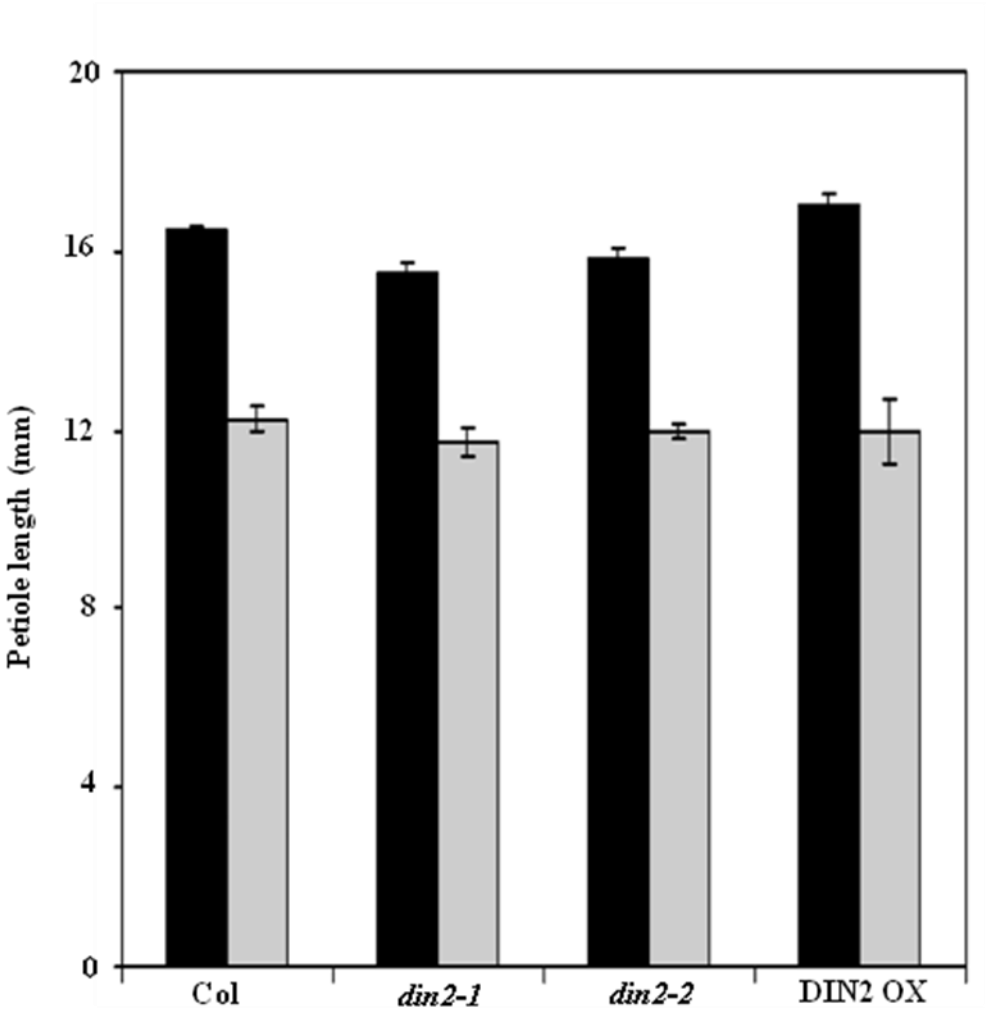


Figure 4.2.8.4. Petiole length of *din2* mutants, DIN2 over-expresser and wild-type (Col) plants were grown for 14 days in high R:FR ratio 12 light/ 12 h dark and then they were moved to low R:FR ratio light dark cycles for a further 7 days (grey bars). Controls remained in high R:FR ratio light dark cycles (black bars). Petiole length was measured at the end of the 7 day treatment. A sample size of 15 was used for each genotype.

4.2.8.5. The effect of reduced R:FR ratio on leaf area in din2 mutants and over-expressers

In response to low R:FR ratio, wild-type plants display a decrease in leaf area. This physiological alteration is thought to be a survival mechanism whereby all available resources are channelled into more critical responses such as reproduction.

To investigate the role of *DIN2* in leaf growth, wild-type, *din2* mutants and DIN2 over-expresser seedlings were germinated (as described in section 2.1.3.) and grown in 12 h light/ 12 h dark for 14 days. Half of the plants were then transferred to low R:FR ratio light dark cycles, whilst the controls remained in high R:FR ratio light dark cycles for a further 7 days, leaf area of leaf 5 was then measured (section 2.2.7.). Figure 4.2.8.5. shows that in high R:FR ratio conditions wild-type, *din2-1*, *din2-2* and DIN2 over-expressers all have approximately equivalent leaf areas. When these plants were subjected to low R:FR ratio they all displayed a similar decrease in leaf area. The retention of a reduction in leaf area in the *din2* mutants and *DIN2* over-expressers indicates that *DIN2* either does not participate in this physiological response or that this gene plays a functionally redundant role.

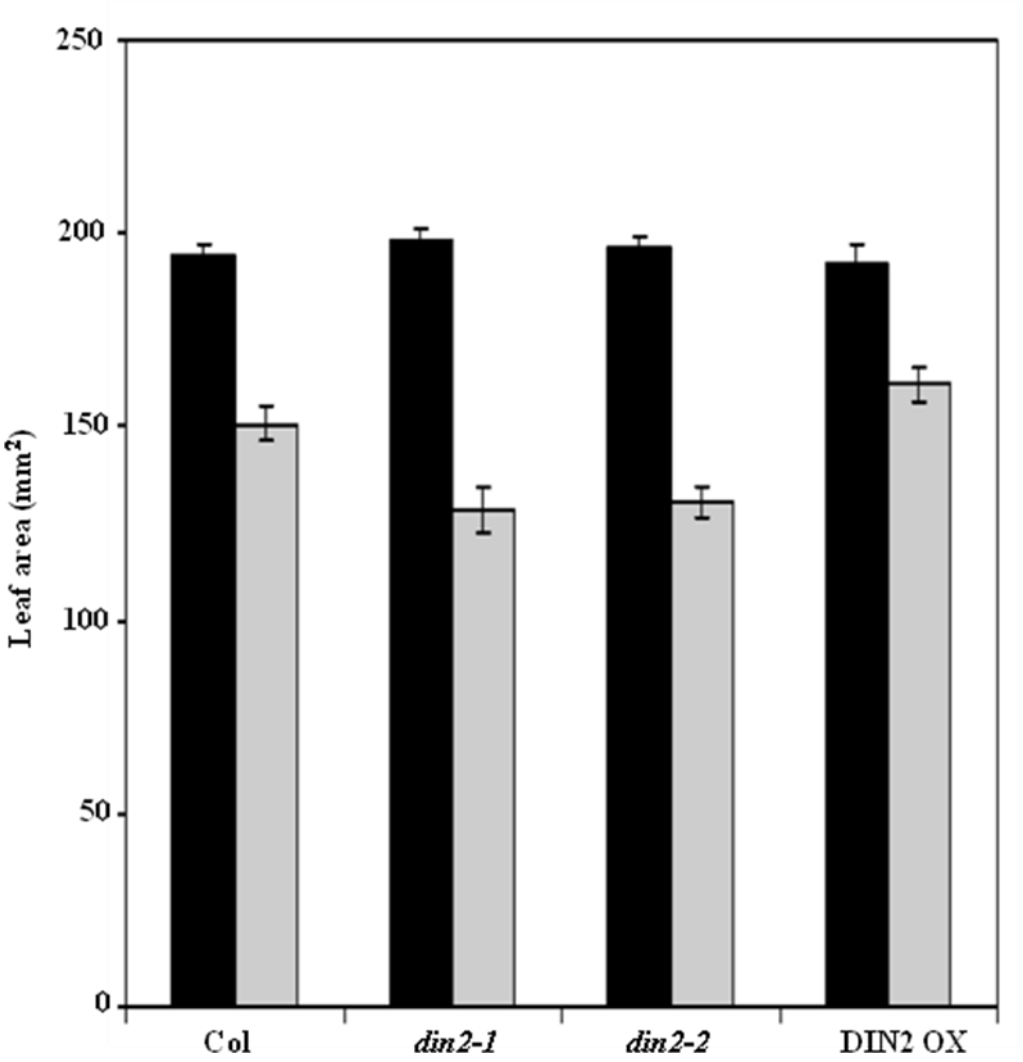


Figure 4.2.8.5. Leaf area of *din2* mutants, DIN2 over-expressers and wild-type (Col) plants grown for 14 days in high R:FR ratio 12 light/ 12 h dark and then they were moved to low R:FR ratio for a further 7 days (grey bars). Controls remained in high R:FR ratio light dark cycles (black bars). Leaf area was measured at the end of the 7 day treatment. A sample size of 15 was used for each genotype.

4.2.8.6. Low R:FR ratio induced acceleration of flowering in din2 mutants and over-expressers

Flowering time is known to be dramatically altered when plants are subjected to a prolonged period of low R:FR ratio light. This response is regulated by the action of phyB, phyD and phyE. In addition to light quality, many other environmental cues, such as seasonal, control flowering time in Arabidopsis. Recently, progress has been made in defining some of the components that are involved in triggering the acceleration of flowering by low R:FR ratio, an example being the correlation of early flowering *phyB* and elevated expression of *FT* (Halliday *et al*, 2003).

To examine the effect of *DIN2* deficiency on the flowering response wild-type, *din2-1*, *din2-2* and DIN2 over-expressers were germinated (see section 2.1.3.) and grown in 12 h light/ 12 h dark high R:FR ratio cycles for 7 days. Seedlings were transplanted into soil trays (2.2.4.). Seedlings were then transferred to low R:FR ratio light dark cycles, where they remained until bolting. Controls remained in high R:FR light dark cycles. All measurements were made at bolting, and the data represents the mean from 15 plants. Flowering time was recorded using rosette leaf number. Rosette leaves were readily distinguished from axillary leaves on the basis of their morphology. Figure 4.2.8.6. shows that wild-type plants show a pronounced acceleration in flowering under low R:FR ratio light. Similarly, *din2* mutants and over-expressers behaved in a comparable way. Interestingly the *din2* mutants were found to have a large number of axillary leaves present at bolting (figure 4.2.8.6.). This is discussed further in section 4.2.8.7. In conclusion *DIN2* does not seem to play a fundamental role in the R:FR ratio mediated acceleration of flowering.

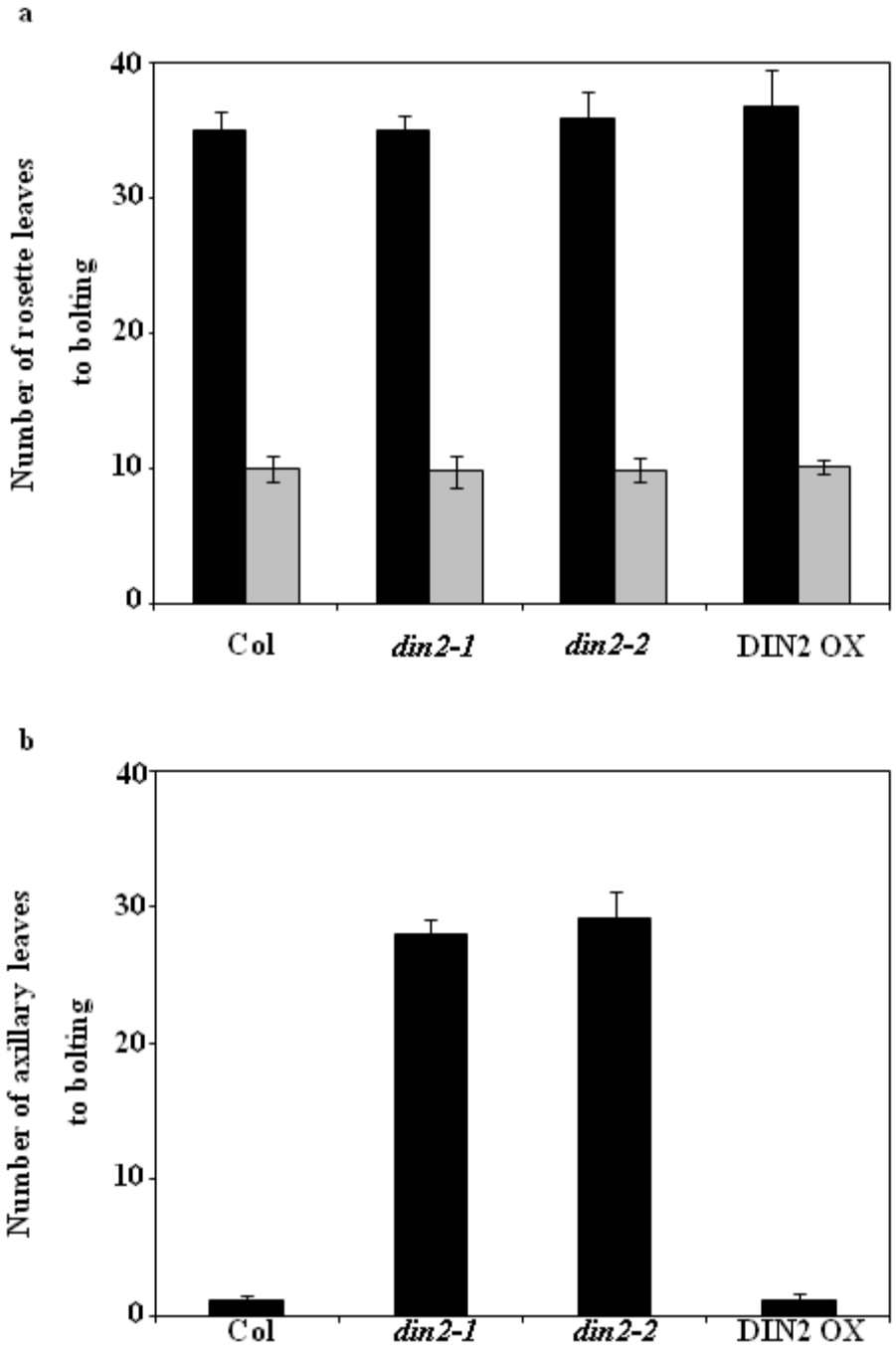


Figure 4.2.8.6. a. Wild-type (Col), *din2* mutants and DIN2 over-expressers were grown for 7 days 12 h light/ 12 h dark for 7 days. The seedlings were then moved to 12 h light/ 12 h dark low R:FR ratio conditions or they remained in high R:FR ratio. The number of rosette leaves were then counted at the onset of bolting. A sample size of 15 was used for each genotype. b. The number of axillary leaves present at bolting.

4.2.8.7. Axillary leaves of din2 mutants and over-expressers

Plants are able to alter their shoot system in response to environmental change. The actual structure of the shoot system is derived from the activity of the primary shoot apical meristem. This branching architecture, although plastic to environmental cues, is genetically determined. Two developmental events underlie the shoot branching process: formation of axillary meristems in the leaf axils and growth of axillary buds. The growth of axillary meristems is inhibited by primary inflorescence; a phenomenon known as apical dominance. It is thought that auxin and cytokinin have a major role in controlling this process (Phillips, 1975; Cline, 1994; Tamas, 1995; Napoli *et al*, 1999). Auxin has an inhibitory effect on the growth of axillary bud outgrowth whilst cytokinin promotes it. More recently genes involved in the axillary branching pathway have been identified; *MAX1* and *MAX2* (*More Axillary Branching 1 and 2*). It is thought *MAX1* and *MAX2* are involved in repressing axillary growth by controlling the rate of cell production in axillary shoot meristems (Bennett *et al*, 2006).

During the physiological experiments described in the previous sections, it was noted that *din2-1* and *din2-2* knockouts showed enhanced axillary leaves at the vegetative stage. To verify this phenotype *din2-1*, *din2-2* and DIN2 over-expressers were grown (section 2.1.3.) with their respective wild-type control under 12 h light/ 12 h dark photoperiods in either high R:FR ratio or low R:FR ratio. At bolting the axillary leaves were dissected from the shoot axis so that axillary leafs could be examined. The *din2* mutants appeared unable to repress the growth of axillary leaf development in high R:FR conditions (figure 4.2.8.7.). Interestingly, in contrast the *DIN2* over expresser line had wild-type axillary leaf architecture in these conditions. Furthermore in low R:FR ratio light, all adult transgenic plants had leaf morphology

similar to wild type. These data suggests that *DIN2* may play a role in repressing the formation of axillary leaves in high R:FR ratio conditions. Thus, it is possible that *DIN2* is part of the molecular pathway involved in the development of axillary leaves: when *DIN2* is not present, 'inhibitory' control of apical dominance is lost.

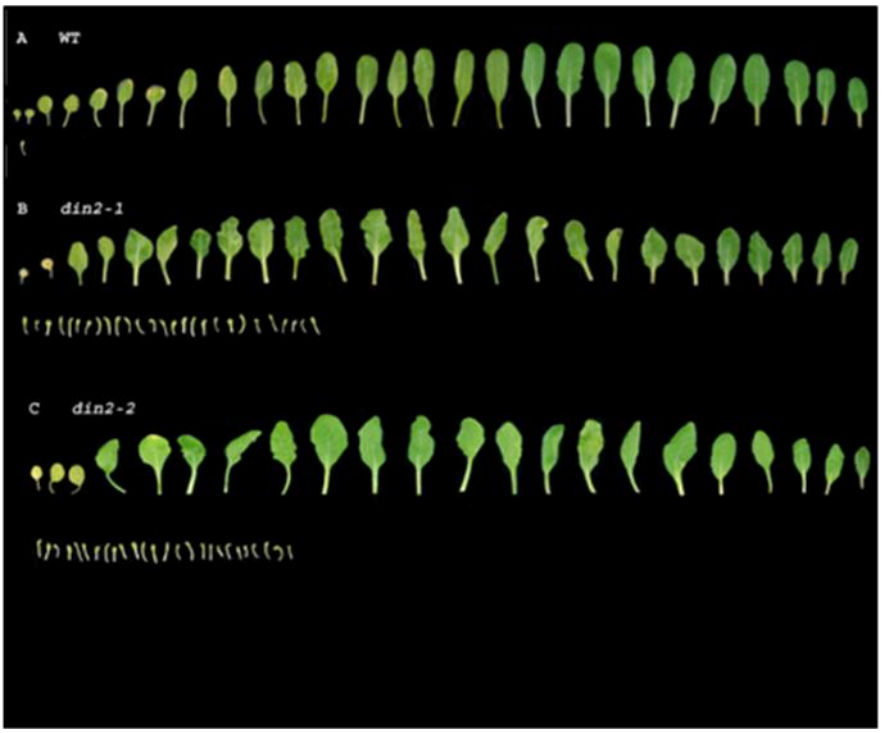


Figure 4.2.8.7. Vegetative shoot development in 12 h light/ 12 h dark high R:FR ratio conditions in *din2-1*, *din2-2* and wild-type (Col). The leaves are laid out in order of emergence at bolting, oldest leaf on the left. All axillary leaves are laid out below. The axillary shoot have been dissected from the shoot axis.

4.2.9 Identification of promoter motifs in *DIN2*

To identify conserved sequence motifs in the promoter region of *DIN,2* a Web Signal Scan Program was used (PLACE). This program identifies previously described promoter region motifs. This analysis permits insight into putative regulators of *DIN2* expression, by detecting protein-recognition elements. For example the promoters of light regulated genes are likely to be responsive to the same pathway and therefore share common regulatory motifs. Table 4.2.9. depicts the relevant light/ sugar regulatory motifs in the promoter region of *DIN2*. The results show that *DIN2* contains many light regulatory motifs. There is also one motif present that is a sugar repressive element.

Elements and related motifs	Sequence	Description of motif
CCAIATLHCBI	AAMAATC	CCA1 binding site; CCA1 protein (myb-related transcription factor) interact with 2 imperfect repeats of AAMAATCT in lhcbi 3 gene of At. Related to phytochrome
GATA box	GATA	Required for light regulated expression
GTI Consensus	GRWAAW	Consensus GT-1 binding site in many light regulated genes
IBOXCOR	GATAA	Conserved sequence of upstream of light regulated genes of both monocots and dicots
INRNTPSADB	YTCACTYY	Initiator elements found in the tobacco psaDb gene promoter without TATA boxes; light responsive transcription of psaDb depends on Inr but no TATA box
PIATGAPB	GTGATCAC	PI found in AT mutations in RT resulted in reductions of light activated gene transcription
SREATMSD	TTATCC	Sugar repressive element
TBOXATGAPB	ACTTTG	Mutations in the TBox resulted in reductions of light activated gene transcription

Table 4.2.9. Light/ sugar regulatory motifs found in 1000 bp of *DIN2* promoter region. Promoter elements found using Web Signal Scan Program (PLACE).

4.3. Discussion

The results detailed in this chapter show that *DIN2* transcription is not only dark induced, but is also R:FR ratio light regulated. Transcript of *DIN2* is seen to increase rapidly after only 30 min of low R:FR light. This rapid flux in *DIN2* transcript suggests that it is a high priority target in the molecular shade avoidance pathway. The low R:FR ratio induction of *DIN2* transcript is rapidly reversed upon receiving high R:FR ratio light. This is likely to be a physiological break: when plants perceive low R:FR ratio, elongation occurs in an attempt for a plant to grow beyond the shade of its neighbours however, once the plant reaches high R:FR ratio light conditions, the shade avoidance response is rapidly reverted through phytochrome conversion. This suggests that *DIN2* is likely to be a necessary component of the shade avoidance response that is repressed in normal daylight conditions.

The phytochromes play a major role in regulating *DIN2* in R:FR light. In Arabidopsis, the perception of a change in R:FR ratio mainly involves the action of phyB and it is this phytochrome, when in its Pfr conformation, that acts to repress *DIN2* in high R:FR ratio conditions. Once a reduction in R:FR ratio occurs then phyB is converted principally to the Pr conformation. One consequence of this appears to be elevation of *DIN2* transcription. There is also clear evidence that phyA plays a role in *DIN2* regulation, as in seedlings lacking functional phyA, *DIN2* is over-expressed when compared to wild-type. This implicates phyA as a regulator of *DIN2* in low R:FR ratio conditions. It is likely that phyA acts to limit *DIN2* transcription in order to attenuate the action of phyB.

The PIF transcription factors are known to recognise and bind to DNA promoter regions that contain a G-Box, this association enables PIFs to directly

regulate transcription of target genes. *DIN2* is unlikely to be a direct target for light regulated PIF transcription factors as it lacks a G-box motif (Matinez-Garcia *et al*, 2000). Therefore, there are likely to be additional intermediate transcription factors within this molecular pathway. There is now sufficient evidence to demonstrate a role for a number of such light regulated transcription factors. Three of these; PIF3, PIF4 and PIF5; have been examined in the current study to determine if they directly influence *DIN2* expression. The DNA-binding bHLH protein, PIF3 is a phytochrome signalling intermediate and has been shown to bind to phyB in a highly Pfr-preferential manner in vitro (Ni *et al*, 1999). The work presented here demonstrates that this signalling intermediate negatively regulates *DIN2* transcription in low R:FR ratio. However, as previously mentioned it is unlikely that *DIN2* is a direct target of PIF3 as *PIF3* /phyB complexes act through G-Box motif binding in the promoter region of target genes. Similarly, the data presented in this chapter indicates that the phyB-binding proteins PIF4 and PIF5 positively regulate the expression of *DIN2* in low R:FR ratio light. It is however again unlikely that these transcription factors directly associate with the *DIN2* gene as it lacks the necessary G-Box motifs in its promoter region. Thus, it is likely that there are as yet unidentified intermediates within this signalling pathway. Future work will help determine what these intermediates are.

It has been suggested that PIF4 and PIF5 induce shade avoidance responses through the regulation of genes involved in growth responses, as plants lacking functional *PIF4* and *PIF5*, although still responsive to a reduction in R:FR ratio, have an aberrant phenotype (Lorrain *et al*, 2007). The gene *SHL*, which is thought to be involved in protein-protein interactions and is associated with transcriptional regulation and chromatin remodelling factors, has also been shown to regulate *DIN2*.

In an SHL over-expresser *DIN2* transcript has been found to increase by over 10-fold (Mussig and Altmann, 2003). Thus known intermediates which both negatively and positively regulate *DIN2* in response to changes in light quality have been described. Both PIF4 and PIF5 have been found to act to positively regulate *DIN2* in the molecular shade avoidance pathway. Conversely, PIF3 has been shown to act to negatively regulate *DIN2* in response to low R:FR ratio conditions and similarly acts to negatively regulate *DIN2* in high R:FR ratio light. It is likely that these transcription factors all feed into a pathway that eventually converges to control *DIN2* transcription.

The work presented above clearly demonstrates that *DIN2* expression is tightly controlled during shade avoidance responses and also by the circadian clock. Interestingly, like many other low R:FR ratio regulated genes (e.g. *PIL1*, *ATHB2* and *ENDO1*), maximal de-repression of *DIN2* occurs at subjective dawn. This is now understood to coincide with the onset of elongation growth. Hypocotyl elongation in day/ night cycles shows a maximum de-repression to low R:FR ratio at around subjective dawn and maximum repression at around subjective dusk (Lorrain *et al*, 2007). One possible explanation is that *DIN2* is involved in mobilising carbohydrates and therefore preparing cells for elongation.

It has been shown that *DIN2* transcript is induced in darkness and can be repressed by exogenous sugar (Fujiki *et al*, 2001). Furthermore, *DIN2* transcription is also induced by a change in R:FR ratio, a response that can also be repressed by exogenous sugars. This is noteworthy as *DIN2* belongs to the β-glucosidase family of enzymes which are likely to function in the utilisation of cell wall polysaccharides as alternate carbon sources under sugar depleted conditions (Fujiki *et al*, 2001). Recent studies demonstrate that a reduction in R:FR ratio causes a depletion of cellular free

sugars (Whitelam, pers com, 2007). This suggests that reduced low R:FR ratio decreases free sugar availability and signals activation of *DIN2* transcription. Whilst an attractive hypothesis, it is unlikely as *DIN2* transcription increases after only 30 min of low R:FR ratio light, far faster than it takes for free sugars to deplete. Despite this, it is possible that low R:FR ratio light presage impending sugar depletion conditions thereby rapidly initiating *DIN2* transcription. Alternately, it could act to utilise β-glycoside conjugates as a carbohydrate source when plants are subjected to a prolonged reduction in R:FR ratio. Such a mobilisation of sugars would therefore allow physiological shade avoidance responses such as elongation growth to occur.

In response to low R:FR ratio *din2* mutants show no altered phenotype when compared to wild-type and this suggests that *DIN2* is unlikely to be essential for the low R:FR ratio responses to occur. It is more likely that if *DIN2* is playing a role in mobilising carbohydrate sources required during growth, it acts in concert with other sugar mobilising genes.

It has been identified that the *din2* mutants when grown in high R:FR ratio conditions show excess axillary leaves which are apparent before bolting. No obvious explanation has been found for this aberrant response. However, it is understood that auxin plays a role in controlling axillary bud out growth. In addition, *max* mutants are also involved in repressing axillary growth by controlling the rate of cell production in axillary shoot meristems (Bennett *et al*, 2006). It is therefore plausible that in plants lacking functional *DIN2*, the pathway responsible for controlling/ repressing axillary growth is altered, resulting in the production of axillary leaves. It is tempting to suggest that the reason this phenotype is not seen in low R:FR ratio conditions is because resources are reallocated to enable early onset of flowering, not leaf production.

5. *XTH15*

5.1. Introduction

Complexity of plant architecture is achieved through dynamic remodelling of the cell wall, a complex structure comprising structural proteins, pectins, lignins and cellulose microfibrils, the latter of which is intimately associated with hemicellulose networks. Xyloglucan is the most abundant hemicellulose in dicotyledonous plants. Xyloglucan polymers interconnect with microfibrils via hydrogen bonds (Fry, 1989; Hayashi, 1989; McCann *et al*, 1990; Pauly *et al*, 1999; Becnel *et al*, 2006) and are thought to act as a tether, giving tension and resistance to the cell wall. As plants avoid shade, it is possible that modifications of Xyloglucan polymers helps to orchestrate cell wall restructuring.

5.1.1. Enzymatic activity of XTH genes

XYLOGLUCAN ENDOTRANSGLUCOSYLASE/ HYDROLASE (XTH) proteins function to endolytically cleave xyloglucan polymers and subsequently join cleaved ends to other xyloglucan chains (xyloglucan endotransglucosylase; XET activity) or to water (xyloglucan endohydrolase; XEH activity) (Becnel *et al*, 2006; Rose *et al*, 2002). The catalytic activity of XTH proteins is thought to loosen cell walls through weakening of the structure that constrains cellulose microfibrils (Hayashi, 1989; Fry, 1989; Hoson *et al*, 1991). The products of the *XTH* gene family are likely to be involved in this process as they act through XET-mediated cleaving and rejoining of xyloglucan chains or suitable xyloglucan derived oligosaccharides, thereby restructuring existing wall xyloglucan (Xu *et al*, 1996; Mauch *et al*, 1997; Nishitani, 1997; Fry, 2004). It is also thought that some XTH members may function in the genesis of vascular secondary wall tissue (Bourquin *et al*, 2002; Matsui *et al*, 2005).

5.1.2. Xyloglucan endotransglucosylases

In Arabidopsis thirty-three open reading frames encoding for XTH protein family members have been identified (Arabidopsis Genome Initiative, 2000; Asamizu *et al*, 2000; Yokoyamama and Nishitani, 2001). Whilst these genes are spread across all five chromosomes, one third are concentrated as clusters consisting of two to four members, a phenomenon that is likely the result of genome duplication and gene reshuffling (Yokoyama and Nishitani, 2001). The *XTH* gene family can be divided into three major subfamilies (Yokoyami and Nishitani, 2001), although no clear diversification of function can be found between each family (Campbell and Braam, 1999a). All subfamily members share several conserved amino acid domains including a hydrophobic amino acid terminus; which has been suggested to function as a signal peptide and an N-linked glycosylation consensus site (Campbell and Braam, 1999b); and a frequently observed DEIDFEFLG sequence which is thought to function as a catalytic site for both hydrolase and transferase activity (Okazawa *et al*, 1993; Campbell and Braam, 1998). In contrast to the N-terminal domain, the carboxy terminal of XTH proteins shows considerable diversity. Differences in this domain determine XTH subfamily segregation (Campbell and Braam, 1999b). It is unclear as to why such a large number of *XTH* genes have evolved in Arabidopsis, but they may have discreet functional roles in response to specific hormonal and environmental stimuli (Becnel, *et al*, 2006).

The spatiotemporal expression patters of all Arabidopsis XTH family members have been studied in considerable detail. Quantitative RT-PCR studies demonstrated restriction of *XTH* gene expression to distinct organs and tissues in all stages of development (Yokoyama and Nishitani, 2001). Different *XTH* family

members are regionally expressed in roots, stems, flowers or siliques. For example *XTH12*, *XTH13* and *XTH14* are predominantly expressed in roots where as *XTH30* and *XTH33* are predominantly expressed in siliques (Yokoyama and Nishitani, 2001). Although the expression of XTH genes has been shown to be regionally diverse, the expression domain of one family member often overlaps with another. It is still not known whether these genes work together or act in a functionally redundant manner to modify cell wall properties.

The expression of *XTH* gene family members has been shown to be regulated by a number of plant hormones such as IAA, giberellic acid, brassinolides and abscisic acid (Yokoyama and Nishitani, 2001). However, whilst hormones induce changes in transcript abundance, they do not alter regional distribution. In addition the finding that *XTH12* and *XTH13*; two family members expressed in roots that are thought to have arisen from a gene duplication event; respond differentially to exogenous hormones suggesting that at least some of the *XTH* paralogs are functionally divergent. Three members of the XTH family have also been found to be touch sensitive *XTH17*, *XTH22* (*TCH4*) and *XTH25* (Yokoyama and Nishitani, 2001). This may be important for plant cell wall remodelling in environmental conditions that cause physical stress to plants (e.g. strong wind).

More recently, Becnel *et al.* (2006) used various *XTH::GUS* reporter fusion transgenes to study expression of different family members during Arabidopsis development. Their results demonstrate that *XTH* gene expression is widespread: at any given stage of development, XTH-linked reporter protein can be seen in all organs. However, the greatest overall XTH expression was reported to occur in young seedlings. This stage marks the developmental period in which pronounced acceleration of growth occurs.

5.1.3. Xyloglucan endotransglucosylase/ hydrolase 15

The gene *XTH15* is a member of *XTH* subfamily two and is thought to share a common ancestor with *XTH16* (Yokoyama and Nishitani, 2001). Becnel *et al*. (2006) have shown via *XTH15::GUS* construct analysis that this gene may play a role in root, vasculature and inflorescence stem growth. Additionally, *XTH15::GUS* expression is greater in dark-grown than in light-grown seedlings. Quantitative RT-PCR analysis of *XTH15* transcription has revealed that transcript is mainly present leaves, stem, flowers and siliques (Yokoyama and Nishitani, 2001). These are presently conflicting reports on *XTH15* expression levels within developing roots: Becnel *et al*, (2006) found high levels of expression in this region whilst Yokoyama and Nishitani (2001) found relatively little expression. Like other *XTH* members, *XTH15* is known to be regulated by hormones. Auxin has been found to downregulate transcription of this gene.

In summary, the aforementioned data point to an expression profile of *XTH15* that suggests a possible role in physiological and morphogenic responses of both seedlings and adult plants to environmental stimuli. However, whilst the spatiotemporal expression pattern of this gene has been examined in detail, the biological role of *XTH15* remains to be determined. Additionally, the environmental (and subsequent molecular) signals that control *XTH15* gene expression await identification.

This chapter details the rapid regulation of *XTH15* expression by changes in R:FR ratio light conditions. Furthermore, it investigates the roles individual phytochromes, blue/ UV-A-light absorbing cryptochromes and transcriptional regulators play in controlling *XTH15* expression. Finally, through generation of *xth15*

mutants and XTH15 over-expressing lines, a possible biological function of this gene is determined.

5.2. Results

5.2.1. A decrease in R:FR ratio causes an increase in XTH15 relative transcript

The gene *XTH15* was initially identified to show an increase in transcript abundance in response to transient low R:FR ratio light (Unpublished Dr K Franklin, 2003). This observation, combined with previous reports that it may play a role in cell wall modification during growth provided the impetus to study a possible involvement of XTH15 in the shade avoidance response.

To confirm that *XTH15* expression was indeed R:FR regulated, wild-type seedlings (section 2.1.3.) were grown in high R:FR ratio 12 h light/ 12 h dark cycles for 5 days. At subjective dawn seedlings were transferred to low R:FR ratio for 1 h whilst controls remained in high R:FR ratio. Tissue was harvested immediately after treatment. This same protocol was carried out at subjective dusk; however, the 1 h low R:FR ratio treatment ended 30 min before lights off. Quantitative RT-PCR was carried out on all samples (Section 2.6.1.2., 2.6.2.6.). The results, depicted in Figure 5.2.1, show that *XTH15* transcript responds strongly to low R:FR ratio light at both time points. Relative *XTH15* transcript abundance increases dramatically after a relatively brief (1 h) pulse of low R:FR ratio light showing clearly that the gene is R:FR ratio regulated. These data suggest that when a change in light quality occurs, *XTH15* is targeted for transcriptional regulation and an increase in expression occurs, inferring that *XTH15* is involved in rapid shade avoidance responses.

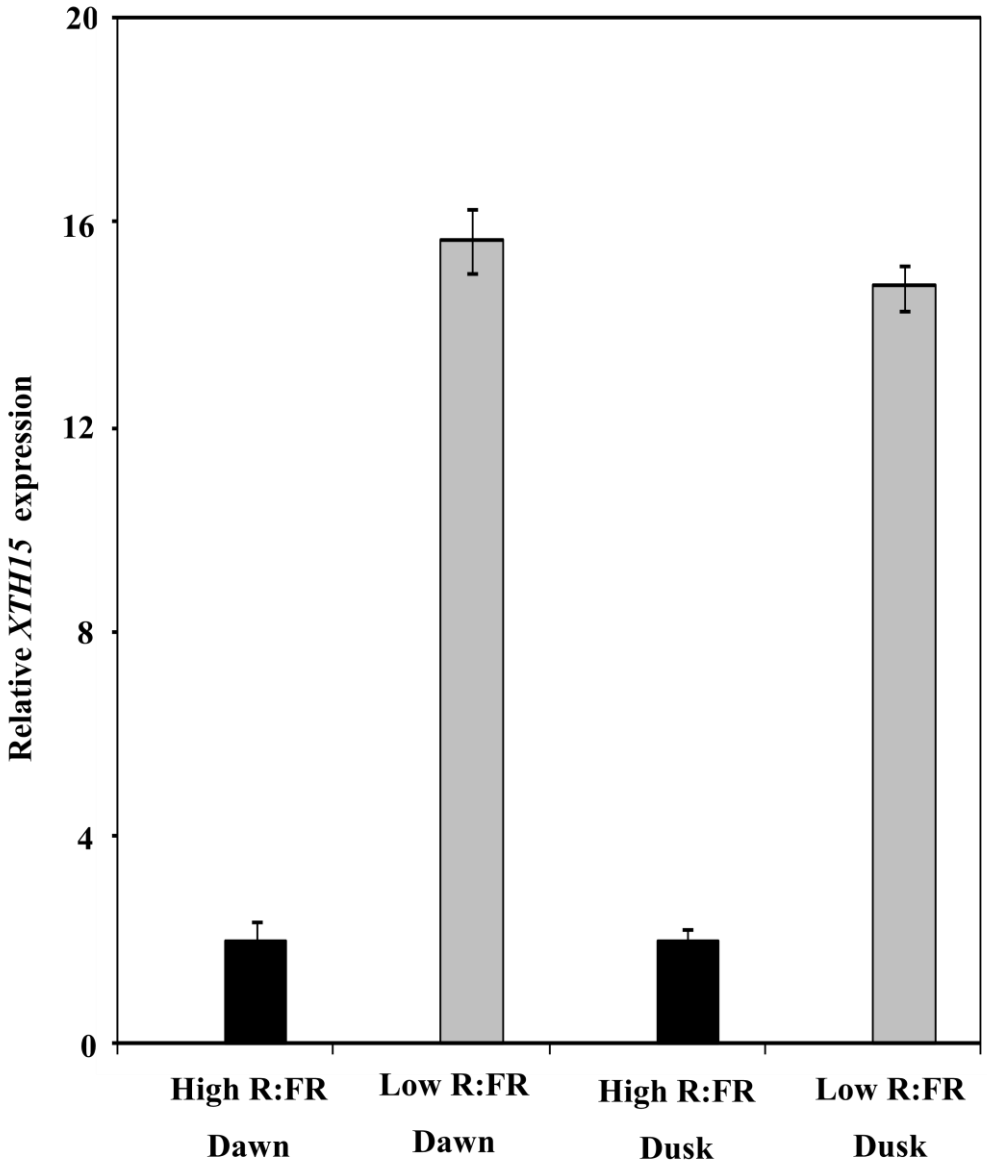


Figure 5.2.1. Analysis of *XTH15* transcript regulation in Columbia seedlings grown at 22°C in response to 1 h low R:FR (grey bars), compared with the control (black bars). The dusk treatment ended 30 min before lights went off. Transcript abundance of *XTH15* was measured using Quantitative RT-PCR. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalised to *ACTIN2*. Relative transcript is normalised to the high R:FR ratio grown control sample.

5.2.2. Kinetics of XTH15

The identification of genes rapidly regulated by low R:FR light allows insight to be gained into the molecular components mediating the rapid shade avoidance response. To investigate whether changes in *XTH15* expression occur rapidly after low R:FR ratio light exposure, and to determine whether any such changes are maintained in prolonged exposure to low R:FR ratio light, wild-type seedlings were germinated (see section 2.1.3.) and grown for 5 days under high R:FR 12 h light/ 12 h dark photoperiods. They were then transferred to low R:FR ratio for 32 h whilst controls remained in high R:FR ratio. Relative transcript was analysed at various time points using Quantitative RT-PCR (Section 2.6.1.2., 2.6.2.6.). As Figure 5.2.2.a demonstrates, elevated *XTH15* transcript abundance is seen after only 15 min R:FR light exposure. Peak transcript levels occur 4 h after onset of this lighting regimen before subsequently declining relatively rapidly. The remarkably fast (*ca.* 15 min) kinetic induction of *XTH15* by low R:FR ratio suggests that it is likely to be a key gene in the molecular pathway controlling shade avoidance. Interestingly, stem/ hypocotyl elongation responses to low R:FR ratio light can be seen after only minutes (Morgan *et al*, 1980; Child and Smith 1987), making it tempting to suggest that *XTH15* could be involved in this process.

The shade avoidance response is rapidly mediated through phytochrome photoconversion. Genes regulated by low R:FR ratio light show a reverse in gene expression levels upon high R:FR ratio light exposure (Morelli and Ruberti, 2002; Sessa *et al*, 2005). To test whether *XTH15* transcript is regulated in this manner, an experiment was performed as detailed above with the exception that after 2 h of low R:FR ratio light, seedlings were reverted to high R:FR light for a further 28 h. Figure 5.2.2.b shows that returning the seedlings to high R:FR ratio results in a rapid

decrease of *XTH15* transcript. This strongly suggests that expression is regulated by phytochrome: Photoconversion of phytochrome in response to a change in light quality would cause a de-repression/ repression of *XTH15* transcript. The rapid low R:FR ratio increase in *XTH15* expression is reversed on perception of high R:FR ratio. This rapid kinetics and reversibility of this response are, broadly speaking, consistent with those of the elongation growth response (Carabell *et al*, 1996; Salter *et al*, 2003).

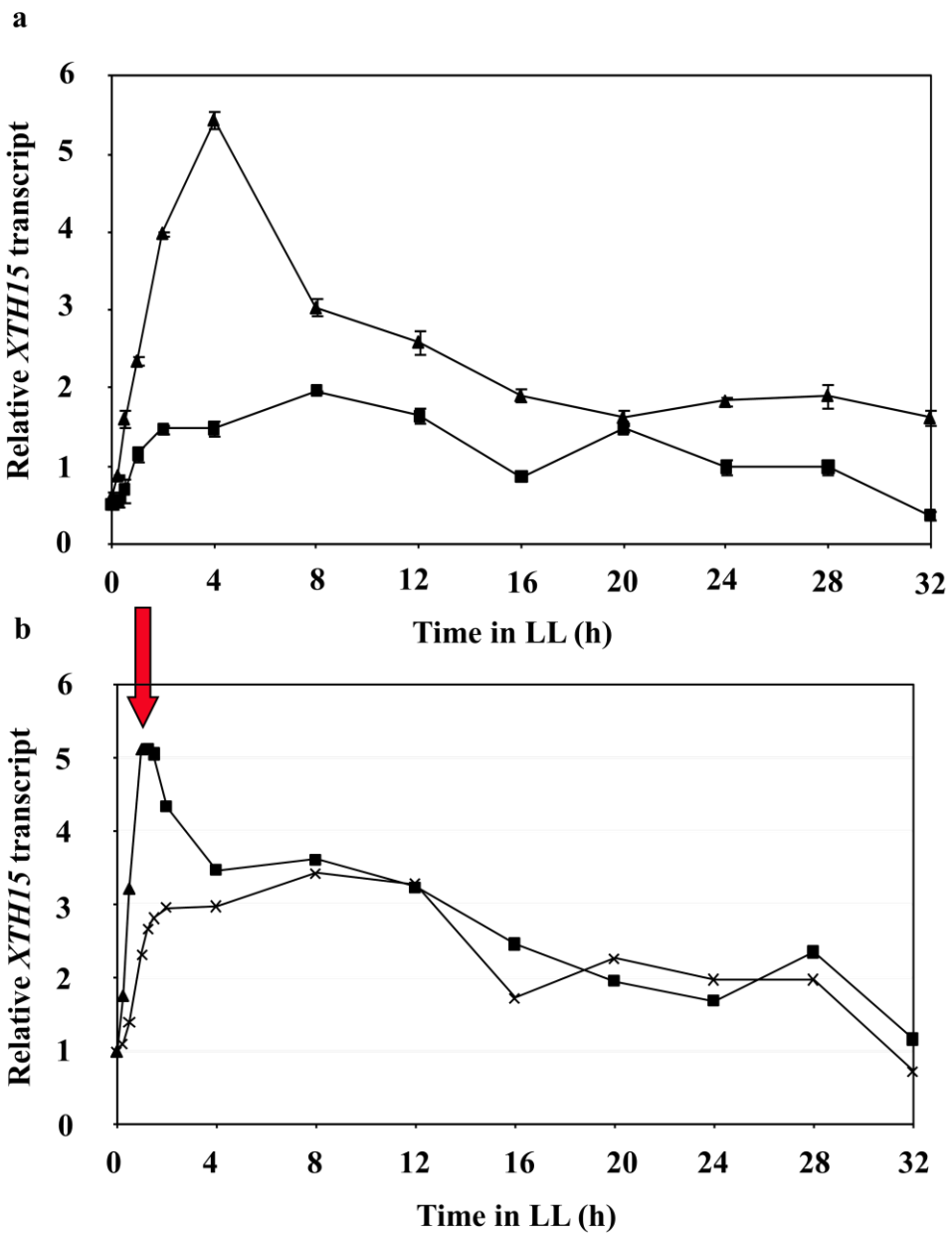


Figure 5.2.2. a Quantitative RT-PCR of *XTH15* transcript in response to low R:FR ratio in wild-type. a. Rapid time course showing the increase in *XTH15* transcript abundance in seedlings transferred to continuous low R:FR ratio (▲) and the control seedlings which remained in high R:FR ratio (■). Previous to the time course seedlings were grown for 5 days under high R:FR 12 h light/ 12 h dark photoperiods. b. Time course of *XTH15* transcript in seedlings transferred to low R:FR ratio light treatment for 2 h (▲) and then the seedlings were transferred back to high R:FR ratio (red ■) for a further 30 h. The arrow indicates the time point at which the seedlings were moved back into continuous high R:FR ratio. Control seedlings remained in continuous high R:FR ratio (x). Previous to the time course seedlings were grown for 5 days under high R:FR 12 h light/ 12 h dark photoperiods. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalised to *ACTIN2*. Relative transcript is normalised to the high R:FR ratio grown control sample.

5.2.3. Phytochrome regulation

It is well known that the perception of a change in the ratio of R:FR is detected through the phytochrome system and that this acts to signal the presence of nearby vegetation that is competing for optimal light conditions.

5.2.3.1. Phytochrome B regulation of XTH15

To analyse the precise function of phytochromes in regulation of *XTH15* expression, phytochrome deficient mutant seeds were germinated as described in section 2.1.3. and grown in high R:FR ratio 12 h light/ 12 h dark photoperiods. On day 5, tissue was harvested and analysed (Section 2.6.1.2., 2.6.2.6.). Subsequent Quantitative RT-PCR of this tissue (figure 5.2.3.1.a) revealed that of all phytochrome family members, phyB is the predominant repressor of *XTH15* expression. Furthermore, constitutively high levels of *XTH15* are observed in *phyB* mutants. This observation is of particular interest as *phyB* mutants exhibit an elongated phenotype indicative of active shade avoidance under similar experimental conditions. Thus it is possible that XTH15 underpins cell elongation growth responses in shade conditions. The following model could be proposed: In high R:FR ratio, phyB is largely in its biologically active conformation which represses *XTH15* whilst in low R:FR ratio light conditions phyB exists mostly in the inactive conformation, enabling de-repression of *XTH15* transcription, putatively resulting in promotion of elongation growth. *XTH15* relative expression is significantly reduced in the *phyD* and *phyE* mutants suggesting that in these monogenic phytochrome mutants, *XTH15* is repressed.

5.2.3.2. Phytochrome A regulation of XTH15

Phytochromes A and B act antagonistically: A single pulse of R light, even when given every day, leads to inhibition of hypocotyl elongation. However, a pre-treatment of continuous FR, which is perceived by phyA, enhances the phyB-mediated response to a terminal R versus FR pulse (Casal *et al*, 1997). Arabidopsis *phyA* mutants are elongated when compared to wild-type when grown in low R:FR ratio conditions (Whitelam and Devlin, 1997). Therefore phyA is thought to temper extension growth of emerging seedlings, thereby preventing exaggerated shade avoidance responses which are predominantly mediated by phyB under high R:FR ratio conditions (Smith *et al*, 1997).

To determine whether PhyA activity influences *XTH15* expression, wild-type and loss of function PHYA mutant seedlings were germinated (section 2.1.3.) and grown in 12 h light/ 12 h dark low R:FR ratio photoperiods for 5 days. Tissue was then removed and subjected to Quantitative RT-PCR analysis (Section 2.6.1.2., 2.6.2.6.). Figure 5.2.3.1.b demonstrates that relative *XTH15* expression is elevated in p*hyA* mutants when compared to controls. This suggests that phyA normally inhibits *XTH15* transcript in low R:FR ratio conditions. It is likely that phyA antagonises phyB-mediated de-repression of *XTH15* to prevent excess mediated XET-like activity, thus avoiding exhaustion of seedling resources.

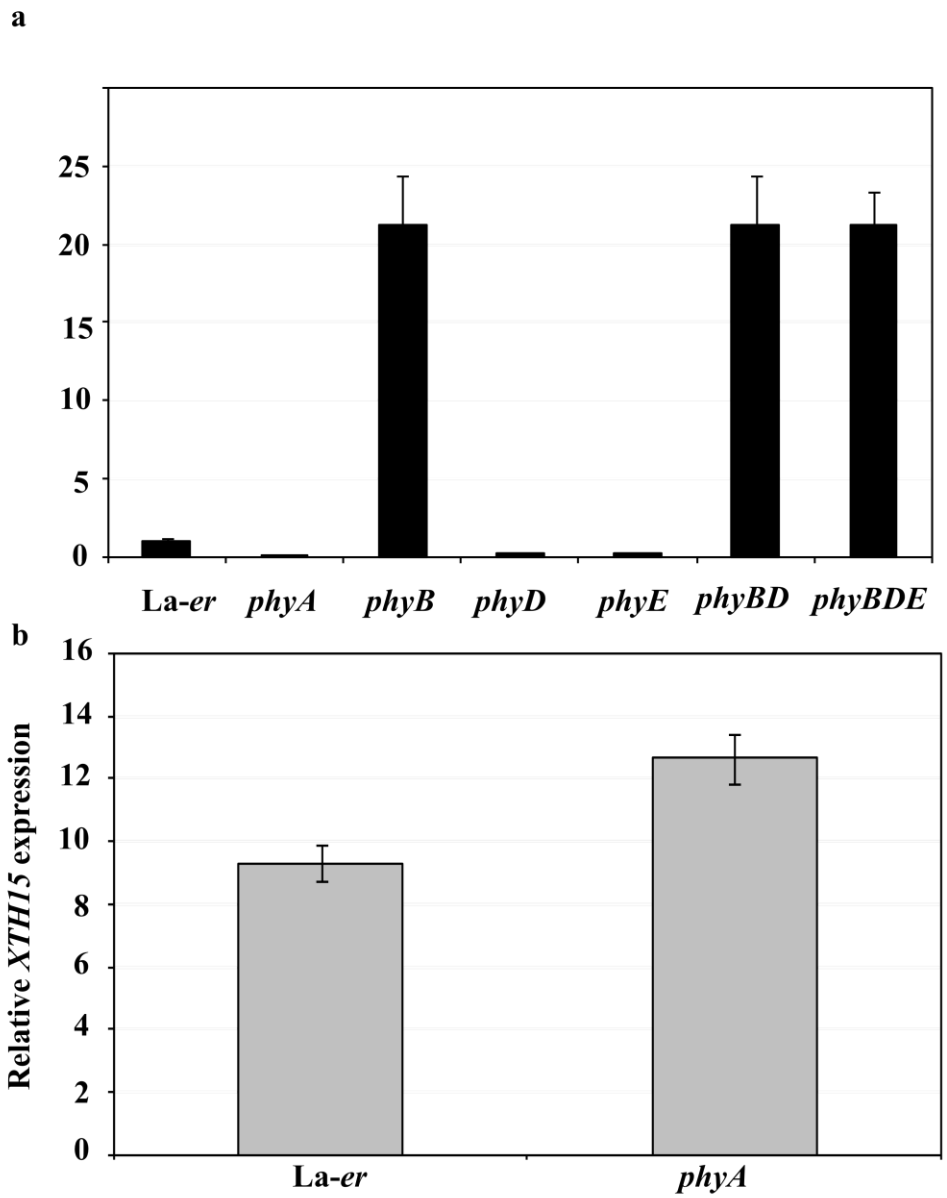


Figure 5.2.3.1. Phytochrome-regulation of *XTH15* transcript abundance. a. Transcript abundance of *XTH15* was measured in 5 day old phytochrome mutants grown in 12 h light/ 12 h dark cycles at 22°C using Quantitative RT-PCR. Relative transcript abundance is normalized to the wild-type control. b. Relative expression of *XTH15* transcript in response to 1 h low R:FR ratio light treatment commencing at dawn. Relative transcript expression is normalised to *XTH15* levels in high R:FR ratio grown la-*er* grown plants. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalized to *ACTIN2*.

5.2.4. Transcriptional regulators of *XTH15*

Changes in R:FR ratio light conditions, alter the confirmation of phyB which triggers a molecular cascade that includes promotion of *XTH15* expression. The rapidity of the *XTH15* transcriptional response to transient low R:FR ratio light is demonstrated in the previous section. Through mutant analysis of known light-regulated transcription factors, the following experiments aimed to determine the principal transcriptional regulators of the *XTH15* in response to low R:FR light.

5.2.4.1. Phytochrome interacting factor 3 regulation of XTH15

Under *in vitro* conditions, the bHLH DNA-binding protein PIF3 is a high-order light-regulated transcription factor that associates with phyB in a highly Pfr-dependent manner (Ni *et al*, 1999). Upon interaction with this active phytochrome isoform, expression of light regulated genes is altered. It is therefore possible that *XTH15* is one of the downstream targets of PIF3.

In order to determine if this was the case, loss of function *pif3* mutant seedlings, PIF3 over-expressing lines and the relevant wild-types were grown (see section 2.1.3.) in high R:FR ratio 12 h light/ 12 h dark for 5 days. On day 6, all seedlings were given a 1 h transient pulse of low R:FR ratio light except controls which remained in high R:FR ratio light. Subsequent Quantitative RT-PCR (Section 2.6.1.2., 2.6.2.6.) of harvested tissue showed that wild-type seedlings display elevated *XTH15* transcript levels following the low R:FR ratio treatment (Figure 5.2.4.1.). In the *pif3* mutants, although *XTH15* transcription is elevated in response to low R:FR ratio light, the induction of *XTH15* expression is slightly blunted when compared to wild-type. In contrast, PIF3 over-expressers had significantly higher levels of *XTH15* relative expression in response to low R:FR ratio light. These findings indicate that

PIF3 positively regulates *XTH15* in response to low R:FR ratio. However, as the promoter region of this gene does not have a known G-box DNA motif (which would be expected to be necessary for a direct PIF3-*XTH15* interaction), it is likely that PIF3 does not directly bind to the *XTH15* gene, but instead acts upstream to alter expression of its transcriptional regulators. Interestingly, *PIF3* does not just regulate *XTH15* in response to a reduction in R:FR ratio light: In high R:FR ratio light conditions, *XTH15* relative transcript is also elevated in the Columbia *pif3* mutant when compared to wild-type. This suggests that *PIF3* also negatively regulates *XTH15* in high R:FR ratio light conditions.

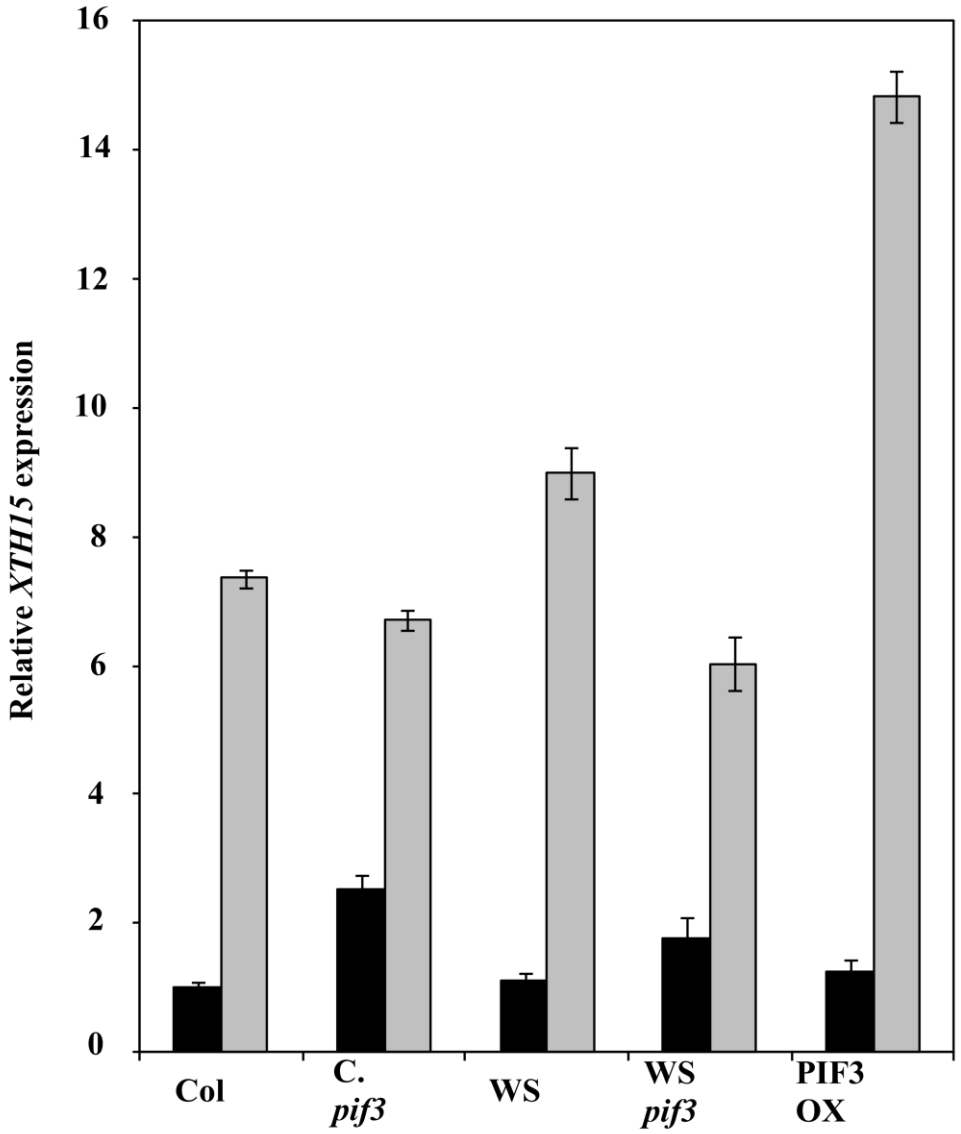


Figure 5.2.4.1. Transcript abundance of *XTH15* was measured using Quantitative RT-PCR in 5 day-old *pif3* mutants and over-expressers in response to 1 h low R:FR ratio treatment at dawn (gray bars). Controls remained in high R:FR ratio (black bars). All seedlings were grown in 12 h light /12 h dark cycles at 22°C. Each value is the mean of 3 separate quantitative RT-PCR reactions normalised to *ACTIN2*. Relative transcript abundance is normalised to the high R:FR ratio grown wild-type sample.

5.2.4.2. *Phytochrome interacting factors 4 and 5*

Recently, the transcriptional regulators *PIF4* and *PIF5* have been found to regulate many low R:FR ratio induced genes (such as *PIL1* and *DIN2).* Both PIF4 and PIF5 are known to interact with phyB and positively regulate shade avoidance. As the aforementioned data shows that phyB is also a repressor of *XTH15* expression in high R:FR ratio conditions (see Section 5.2.3.1), it is possible that the PIF4 and PIF5 mediate changes in *XTH15* transcript abundance under low R:FR ratio light conditions.

To determine whether this was the case, *pif4*, *pif5*, *pif4pif5* mutants, full-length PIF5 over expresser, ΔN PIF5 or ΔN PIF4 over-expresser seedlings were grown (see section 2.1.3.) in high R:FR ratio 12 h light/ 12 h dark cycles for 5 days. On day 6 seedlings were given a 1 h pulse of low R:FR ratio (controls remained in high R:FR ratio conditions) and relative transcript was analysed using Quantitative RT-PCR (Section 2.5.1.2., 2.5.2.6.). Figure 5.2.4.2. shows that in *pif4* and *pif5* null mutants, *XTH15* is still responsive to a reduction in R:FR ratio. However, transcript abundance is reduced when compared to wild-type. In the PIF4 over-expresser *XTH15* transcript levels are elevated in both high and low R:FR ratio light treatments. However, a small, residual response remains upon exposure to low R:FR ratio light.

PIF4 and PIF5 over-expressers are constitutively shade avoiding: Transgenic lines show a considerably elongated hypocotyl when compared to wild-type seedlings. Hence, the dramatic increase in *XTH15* transcript observed in these over-expressing lines may underlie cell wall modifications required for the elongated phenotype. Figure 5.2.4.2. also shows that in high R:FR ratio, the monogenic *pif4* and *pif5* mutants have a significantly greater level of *XTH15* relative transcript. Although this suggests that PIF4 and PIF5 negatively regulate *XTH15* transcript in high R:FR

ratio light, the *pif4pif5* double mutant shows a significant decrease in relative transcript in high R:FR ratio conditions. Taken together, the above experiments strongly suggest that *XTH15* is one of the many genes regulated by PIF4 and PIF5.

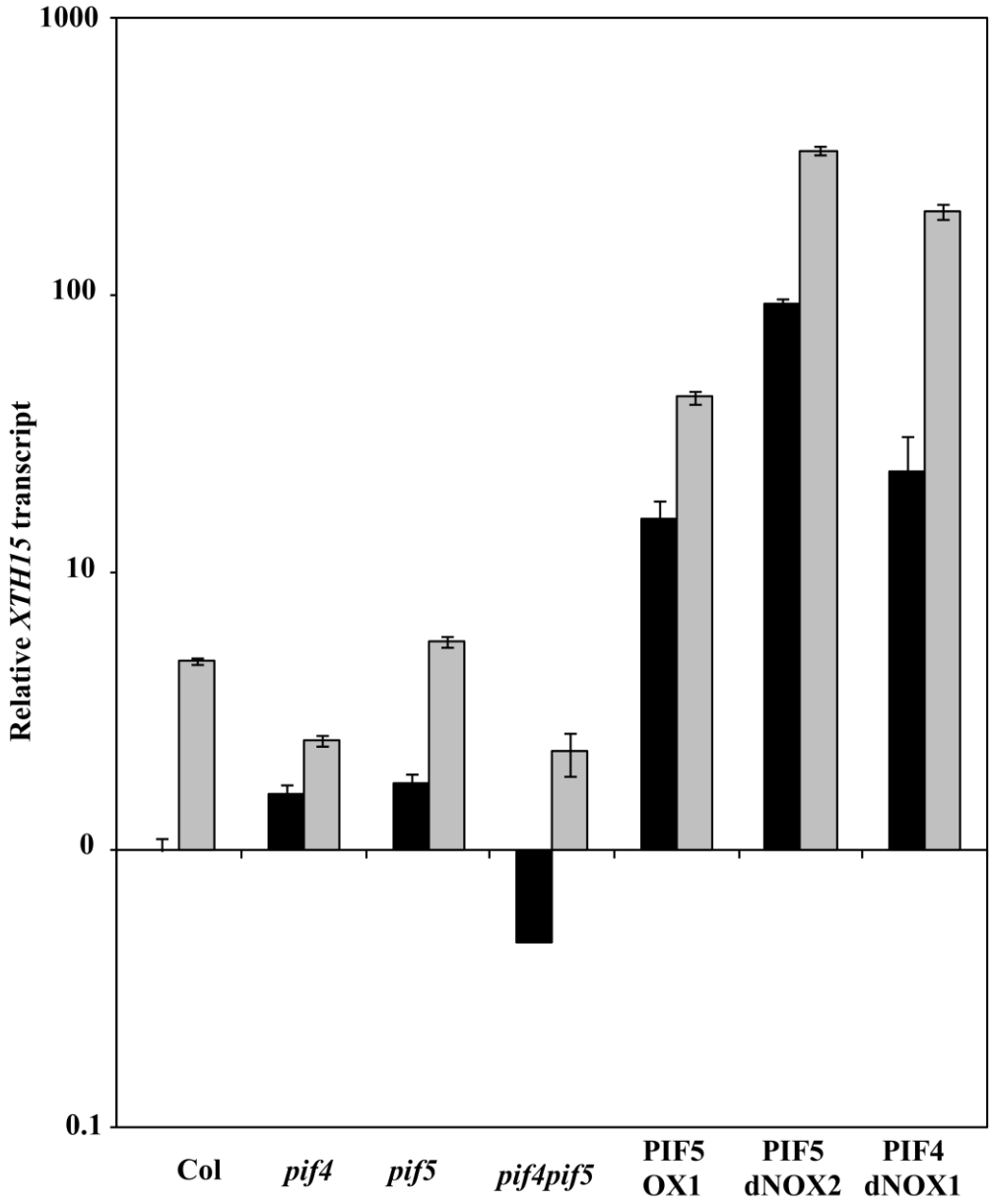


Figure 5.2.4.2. Transcript abundance of *XTH15* was measured using Quantitative RT-PCR in 5 day-old *pif4 and pif5* mutants and over-expressers in response to 1 h low R:FR ratio treatment at dawn (gray bars). Controls remained in high R:FR ratio (black bars). All seedlings were grown in 12 h light /12 h dark cycles at 22°C. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalised to *ACTIN2*. Relative transcript abundance is normalised to the high R:FR ratio grown wild-type sample at dawn.

5.2.5. The circadian clock plays no role in the regulation of *XTH15*

The findings described above clearly demonstrate that *XTH15* is robustly regulated by changes in R:FR ratio light. Recently it has been found that many R:FR ratio regulated genes (such as *PIL1*, *PIL2*, (Salter *et al*, 2003) *DIN2* and *ENDO1* (Unpublished observations G. Whitelam, 2003) are controlled by the circadian clock. All these genes show the greatest de-repression of transcript at around subjective dawn.

To determine whether *XTH15* transcription is also gated by the circadian clock, an initial experiment was conducted whereby wild-type seedlings were given a 1 h pulse of low R:FR ratio at dawn and pre-dusk (figure 5.2.1.). The findings showed that relative *XTH15* transcript levels were comparable at both times of the day. This suggests that *XTH15* is not under the control of the circadian oscillator.

In order to further study any possible regulation of *XTH15* expression by the circadian clock, an experiment was carried out whereby wild-type plants were grown (section 2.1.3.) for 21 days in 12 h light/ 12 h dark high R:FR ratio cycles. Commencing at the onset of dawn on day 22 the plants were moved into continuous high R:FR ratio conditions and at various time points plants were removed and given a 1 h pulse of low R:FR ratio. Quantitative RT-PCR was then carried out (Section 2.5.1.2., 2.5.2.6.). The results, summarised in figure 5.2.5., indicate that although *XTH15* transcript is clearly de-repressed by low R:FR ratio light, no circadian oscillation in transcript abundance occurs in either high or low R:FR ratio light conditions.

To conclude, the above findings demonstrate that whilst *XTH15* expression is indeed regulated by R:FR light conditions, this process is not gated by the circadian

clock. This implies that not all R:FR ratio regulated genes are under the control of the circadian oscillator.

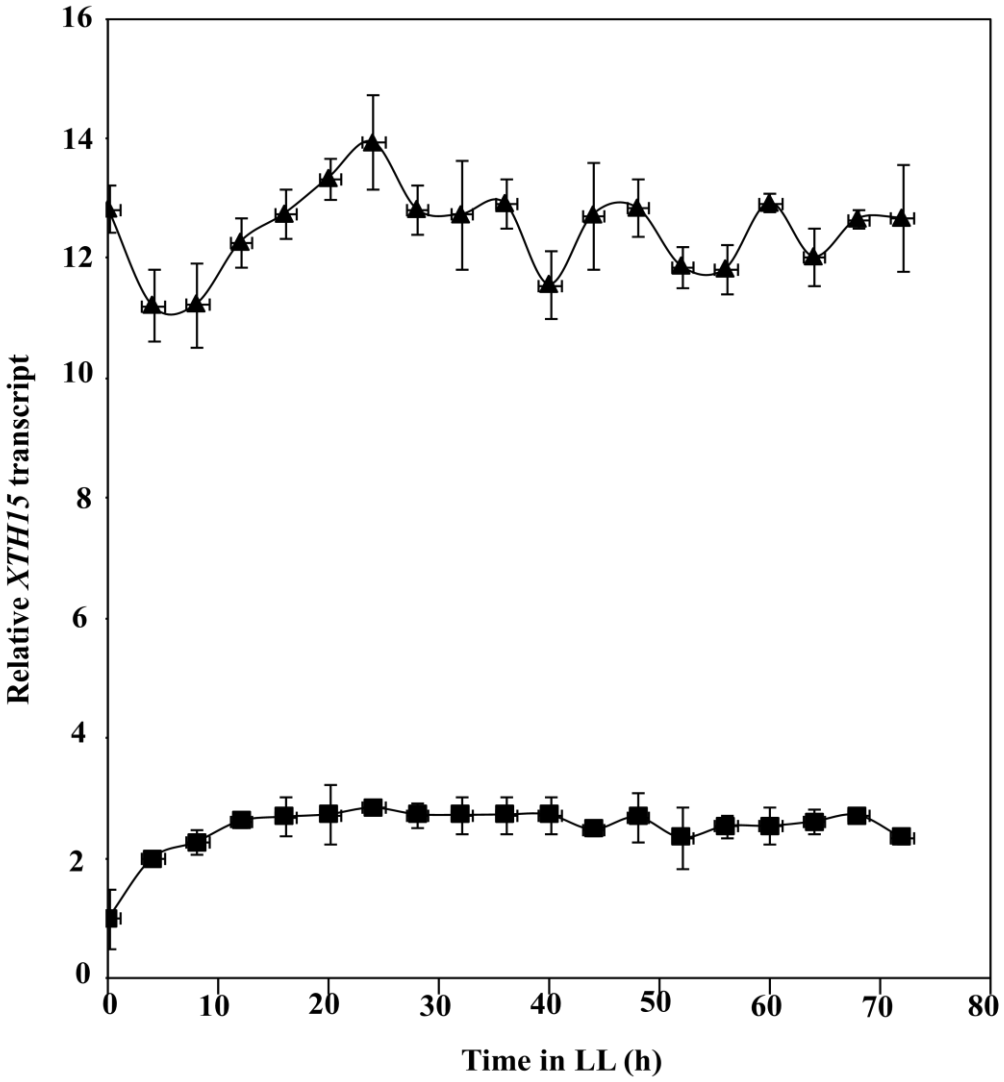


Figure 5.2.5. Circadian analysis of *XTH15* transcript regulation by low R:FR ratio (▲) at 22°C in wild-type plants. Controls remained in high R:FR ratio (■). Plants were grown in 12 h light/ 12 h dark for 21 days, on day 22 they received the various treatments. Transcript abundance of *XTH15* was measured using Quantitative RT-PCR. Each value is the mean of 3 separate reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to the high R:FR ratio grown sample at time 0.

5.2.6. *XTH15* family members

The finding that *XTH15* expression is R:FR ratio regulated raises the possibility that other *XTH* family members are also involved in signalling pathways underpinning the shade avoidance response. Additionally members of the XTH family could act synergistically with *XTH15* to affect development. The following section seeks to address these possibilities.

Due to the large number of genes within the XTH family, a complete analysis of each genes role in shade avoidance was beyond scope of the current study. However, six candidates were chosen for study. Three of these; *XTH3*, *XTH4* and *XTH5*; which are all located in group one; were chosen as they are known to be positively regulated by the brassinolides a hormone which promotes elongation growth (Xu *et al*, 1996; Yokoyama and Nishitani, 2001; Goda *et al*, 2002 and Yin *et al*, 2002). The fourth gene, *XTH16*, was selected as it shares an ancestral gene with *XTH15* and could therefore have a common function and regulatory pathway. The fifth gene, *XTH22*, is known to show an increase in transcript in darkness and, like *XTH3*, *XTH4* and *XTH5*, is also positively regulated by brassinolides. Finally, *XTH24* was chosen as, like *XTH15*, it belongs to group two and again may share a similar regulatory pathway.

The R:FR regulation of each of these genes was examined by growth of wild-type seedlings (as detailed in section 2.1.3) in high R:FR ratio 12 h light/ 12 h dark for 5 days. On day number 6 the seedlings were given a 1 h transient low R:FR ratio pulse. Tissue was then collected and quantitative RT-PCR was carried out (section 2.5.1.2. and 2.5.1.4). The results, shown in figure 5.2.6. demonstrate that *XTH3*, *XTH4* and *XTH5* exhibit a decrease in transcript abundance in response to low R:FR ratio light conditions. Hence under the same light regimen, there is differential

regulation of these genes and *XTH15*. Among the other *XTH* members studied, *XTH16* and *XTH24* appear unresponsive to changes in R:FR ratio light whilst *XTH22* shows a 2-fold increase in transcript following 1h low R:FR ratio light exposure. In addition to these findings, recent work by Allen suggests that *XTH17* transcript also increases 2-fold in low R:FR ratio light (Allen, pers com, 2007).

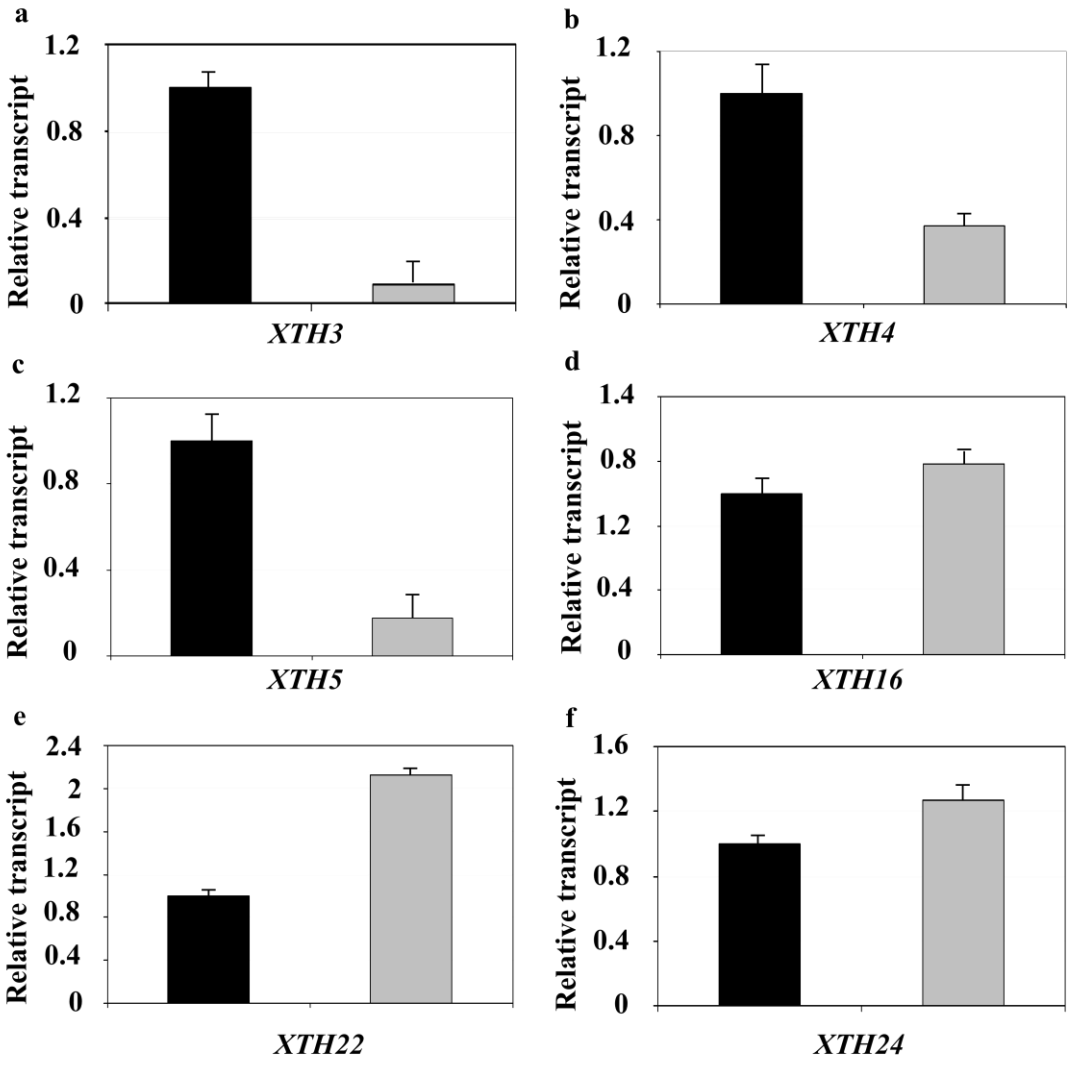


Figure 5.2.6. Quantitative RT-PCR analysis of *XTH* transcript levels following a 1 h treatment of low R:FR (grey bars) or remaining in high R:FR ratio (black bars). The wild-type seedlings were 5 days old grown in 12 h light/ 12 h dark at 22°C. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to wild-type high R:FR ratio sample.

5.2.7. Expression pattern of *XTH15*

As detailed in the Introduction to this chapter, *XTH15* is thought to modify the cell wall through xyloglucan integration and/ or restructuring. Thus it is possible that upregulation of this gene during shade avoidance causes changes in cell wall structure. However, it is not clear where XTH15 is expressed in regions where cell wall restructuring is thought to take place under these conditions.

To gain insight into the precise distribution of XTH15 protein product under different light quality conditions, a GUS transgenic line was obtained from Braam, J. Seedlings of this line were germinated and grown (section 2.1.3.) in either high R:FR ratio or low R:FR ratio 12 h light 12 h dark for 5 days. They were then collected and stained for GUS expression (section 2.3.) Images were taken with a Leica LED2000 microscope and processed in Adobe Photoshop version 7.0. Figure 5.2.7. shows that seedlings grown in high R:FR ratio conditions show no detectable GUS staining whilst those grown in low R:FR ratio conditions show staining in the hypocotyl, particularly around the vasculature. Staining is also present in the primary petioles leading into the cotelydons. Interestingly, the top of the hypocotyl is where the cell walls are modified in order for the seedling to elongate suggesting that *XTH15* is involved in this growth procedure. In the seedling stage, hypocotyls have the greatest overall *XTH15::GUS* expression. This expression pattern is consistent with previous reports that implicate *XTH* in control of cell wall expansion (Becnel *et al*, 2006).

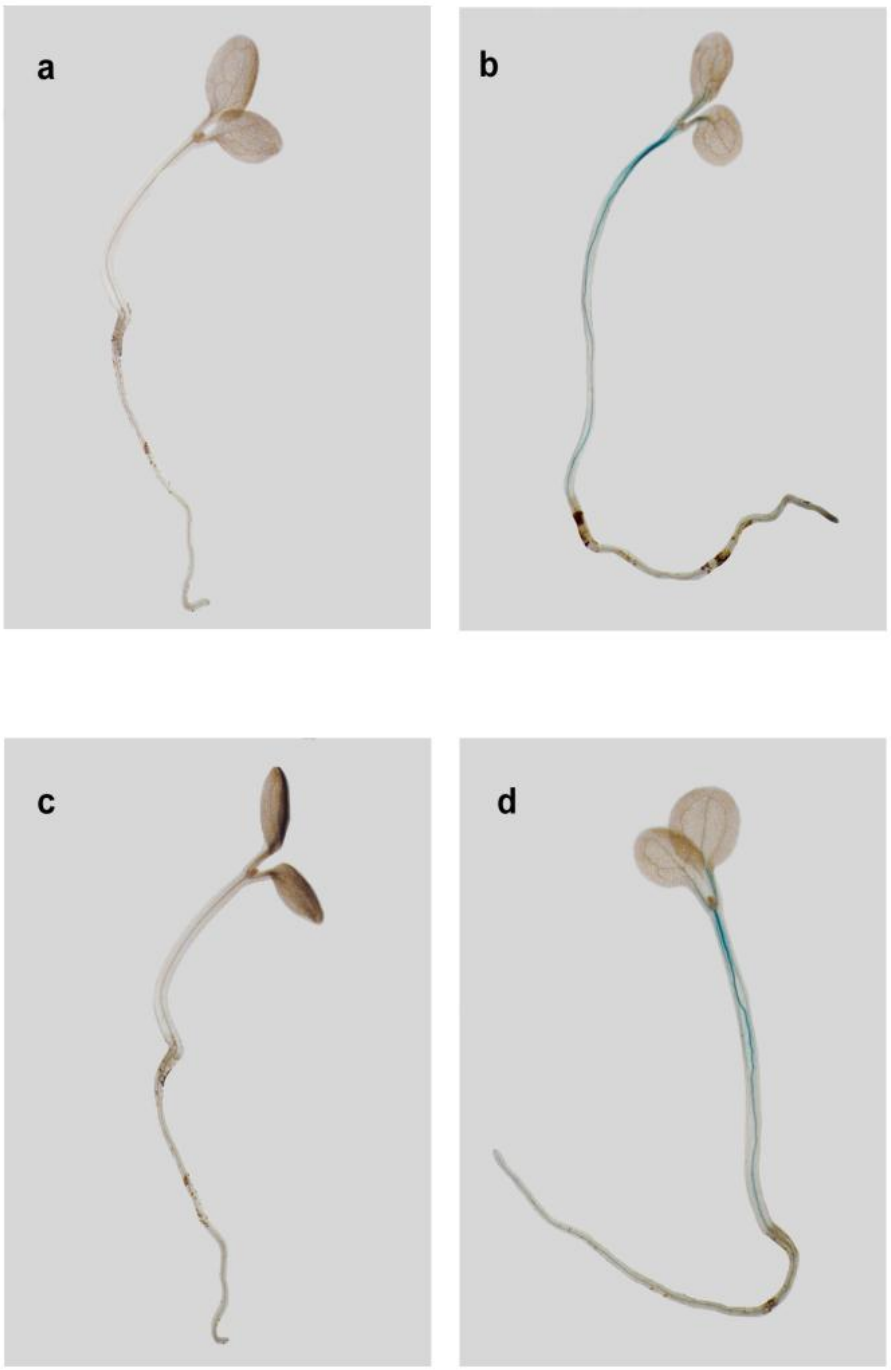


Figure 5.2.7. Localisation of *XTH15::GUS* expression in 5 day old seedlings. a and c. Transgenic seedling grown in high R:FR ratio 12 h light/ 12 h dark for 5 days at 22°C. b and d. Transgenic seedling grown in low R:FR ratio 12 h light/ 12 dark for 5 days at 22°C.

5.2.8. A biological function for *XTH15*

It is thought that members of the *XTH* family are involved in the dynamic remodelling of the cell wall. The ability of *XTH* genes to modify xyloglucan polymers means that these enzymes are likely extremely important for wall architecture modifications. The work presented thus far shows a clear and rapid elevation of *XTH15* transcript in response to a change in R:FR ratio. Given this, it is tempting to suggest that accumulation of *XTH15* transcript is necessary for low R:FR ratio induced hypocotyl elongation. If so, plants lacking functional *XTH15* would be expected to show aberrant cell wall growth in this region. This section seeks to determine if this is the case through analysis of the physiological responses of *xth15* mutants and XTH15 over-expressers in response to transient and prolonged changes in specific light conditions.

5.2.8.1. T-DNA insertion lines

To analyse the biological role of *XTH15* in shade avoidance, two transgenic lines null in *XTH15* were used. These two independent lines; *xth15-1* and *xth15-2*, each homozygous for the T-DNA insertion; were obtained from the Nottingham Arabidopsis stock centre. The knowledge of the T-DNA sequence allowed primers against this region to be designed and this region amplified (see section 2.5.2.4.), enabling verification of homozygous null mutant lines (figure 5.2.8.1.a). Full-length *XTH15* transcript was undetectable in RNA samples extracted from the mutant lines, and RT-PCR analysis also failed to detect any transcript (see figure 5.2.8.1.b). The transgenic lines were then sequenced (section 2.5.2.7.) and the exact location of the T-DNA insertion was established (figure 5.2.8.1.c).

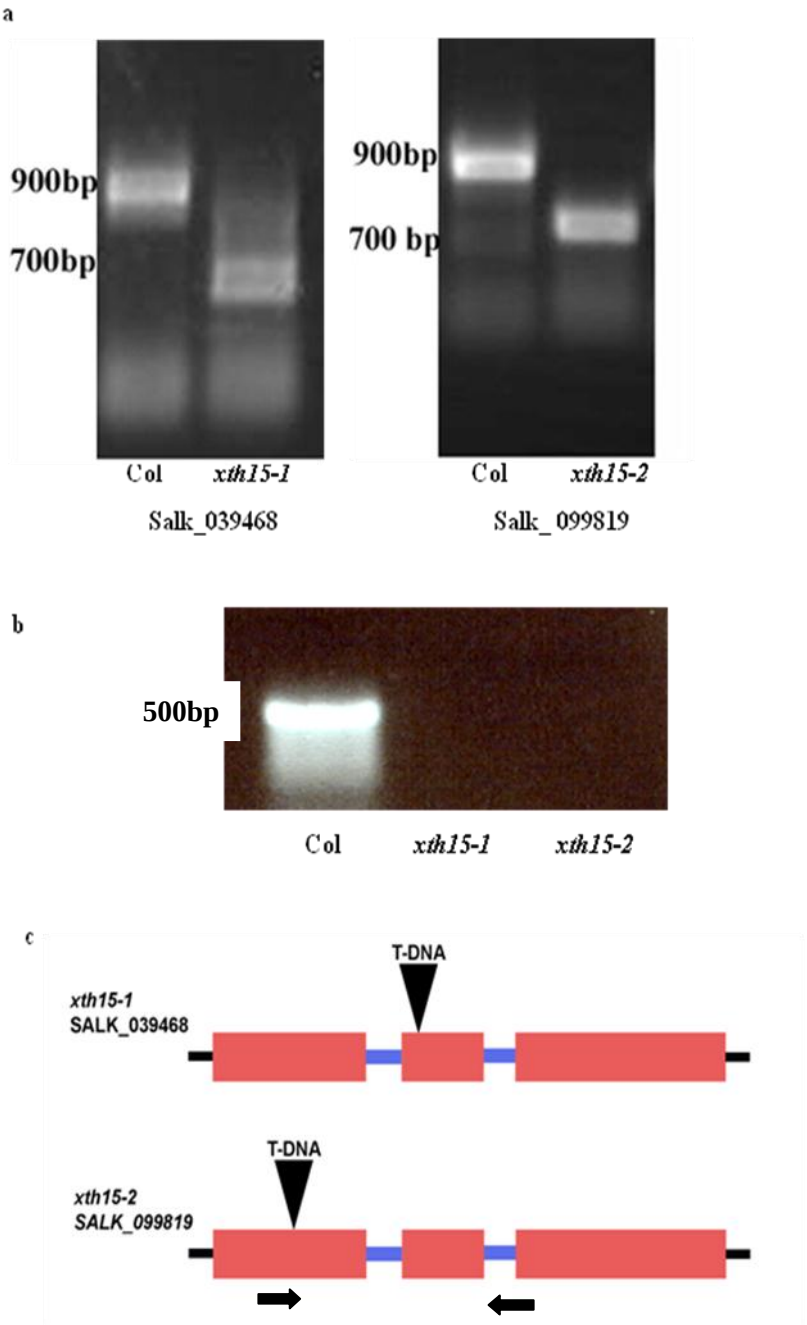


Figure 5.2.8.1. a. Analysis of SALK lines 039468 and 099819. RT-PCR was carried out to verify a homozygous T-DNA insertion in *XTH15*. b. RT-PCR was carried out to detect levels of *XTH15* transcript in *xth15-1* and *xth15-2*. No transcript could be detected. c. Position of T-DNA inserts in *xth15* mutants. The forward reverse arrows represent the forward and reverse primers used in RT-PCR verification.

5.2.8.2. Over-expressing XTH15

Over-expressing lines (XTH15 OX) were constructed in the Columbia ecotype by sub-cloning the *XTH15* cDNA into the plant transformation vector pROK2 under the control of the 35S promoter. Transformation was carried out and transformed lines were selected for according to Kanamycin resistance (40 $\mu g.ml^{-1}$ for additional details, see section 2.1.4). Quantitative RT-QPCR detected a clear over expression of transcript when compared to wild-type (Figure 5.2.8.2.).

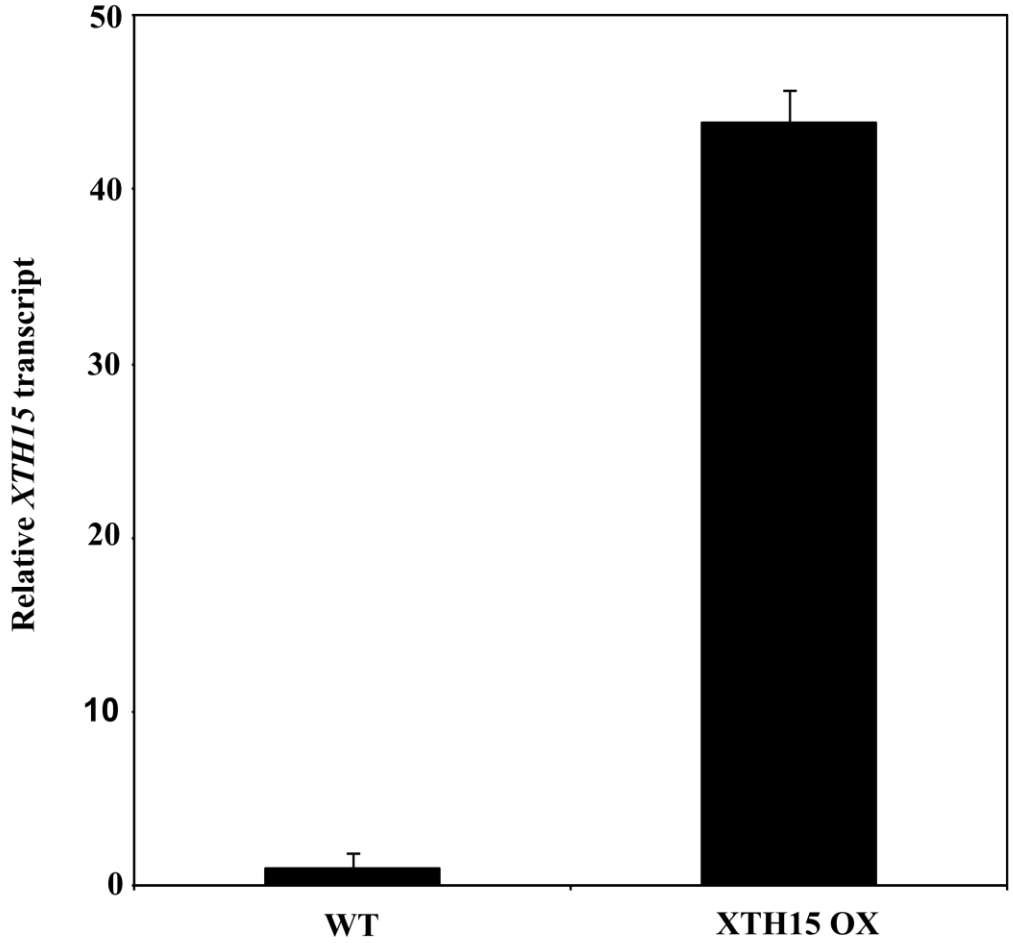


Figure 5.2.8.2. Analysis of *XTH15* transcript in the 35S::XTH15 over expressing construct in low R:FR ratio at 22°C. Transcript abundance of *DIN2* was measured using Quantitative RT-PCR. Each value is the mean of 3 separate reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to the high R:FR ratio grown sample at time 0.

5.2.8.3. Hypocotyl elongation response of xth15 mutants and XTH15 over-expressers

In response to a single 2 h transient low R:FR ratio pulse, a significant promotion of hypocotyl elongation is observed in wild-type seedlings. *XTH15::GUS* reporter fusion constructs show an accumulation of XTH15 protein at the top of the hypocotyl under these conditions (figure 5.2.7.). This is the region where elongation growth is observed. Given this, seedlings either lacking or over-expressing functional *XTH15* may display an abnormal elongation phenotype when compared to wild-type.

To test hypocotyl elongation responses to transient changes in low R:FR ratio light, seedlings lacking functional *xth15* and XTH15 over-expressers were grown (as per section 2.1.3.) for 5 days in 12 h light/ 12 h dark high R:FR ratio light. On day 6 seedlings were given a 2 h pulse of low R:FR ratio which ended 30 min before the onset of subjective dusk, whilst the controls remained in high R:FR ratio light during this period (section 2.2.2.). 24 h later, the seedlings were photographed and hypocotyls measured using ImageJ (see section 2.2.6.). The results (figure 5.2.8.3.a and 5.2.8.3.b) show that in high R:FR ratio conditions all seedlings have a hypocotyl length that approximates that of wild-type plants. However, *xth15* mutant seedlings which received transient low R:FR ratio pulse had an attenuation of hypocotyl growth when compared to wild-type. Interestingly, the results also show that the XTH15 over-expressers when given a 2 h low R:FR ratio pulse show a greater promotion of growth when compared to wild-type. In conclusion this experiment suggests that *XTH15* is an important component of the low R:FR ratio induced hypocotyl growth pathway.

Next, an additional experiment was conducted to examine hypocotyl phenotypes of *xth15* mutants and XTH15 over-expressers in prolonged low R:FR ratio conditions. In this experiment the relevant mutants, over-expressers and wild-

types were germinated and grown (section 2.1.3.) in 12 h light/12 h dark high or low R:FR ratio for 5 days. On day 5 seedlings were photographed and measured using ImageJ (see section 2.2.6.). The results (5.2.8.3.c, 5.2.8.3.d and 5.2.8.3.e) verify the increased inhibition of hypocotyl elongation of the *xth15* mutants in low R:FR ratio conditions when compared to wild-type. The results also confirm that in low R:FR ratio conditions the XTH15 over-expressers show a reduced inhibition of hypocotyl elongation when compared to wild-type. This indicates that *XTH15* plays a role in hypocotyl elongation when R:FR ratio is reduced.

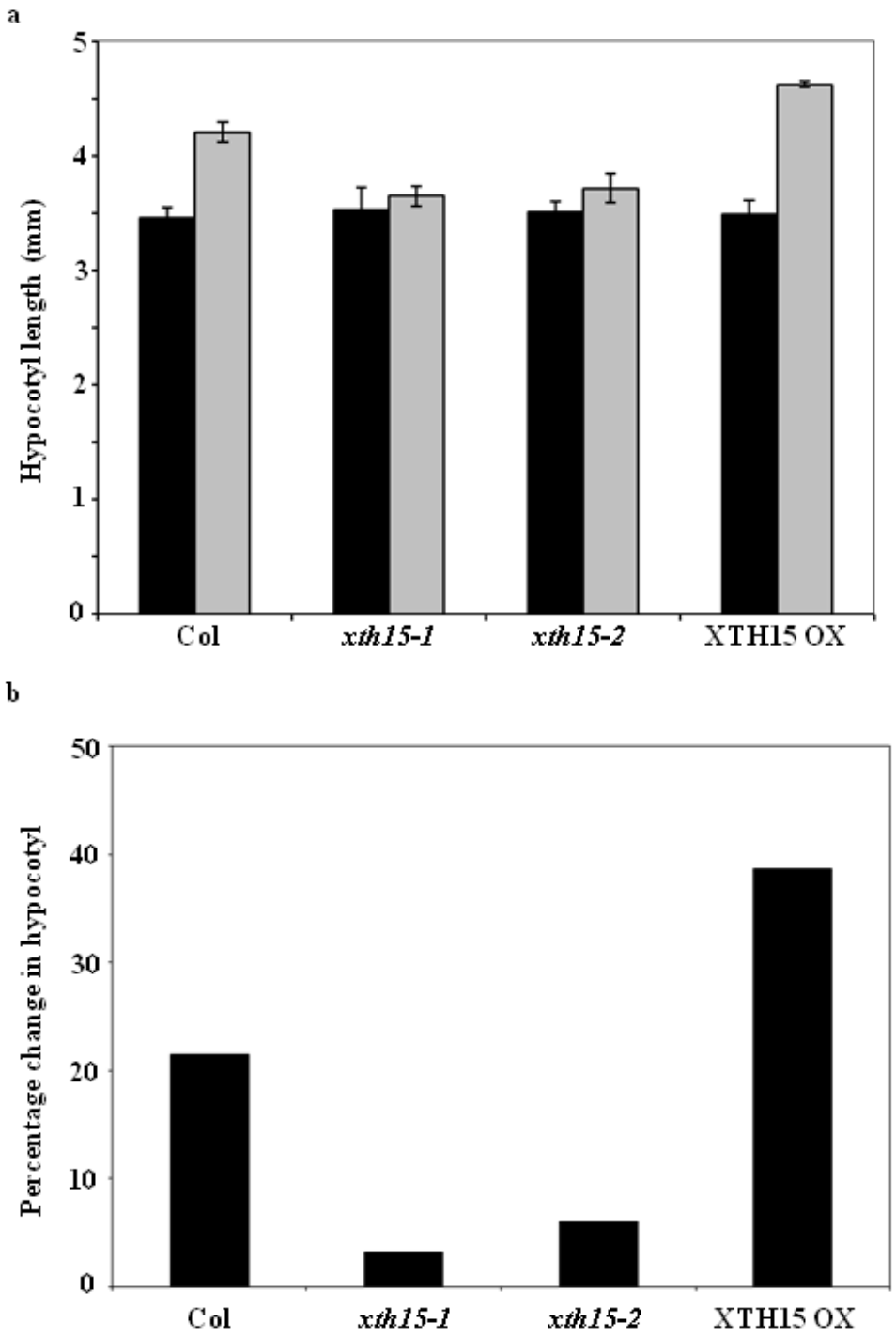


Figure 5.2.8.3. a. Hypocotyl lengths of the relevant wild-types and mutant *xth15* plants grown for 5 days in 12 h light/ 12 h dark cycles at 22°C and treated with single 2 h reduction in R:FR ratio ending 30 min (grey bars) before the end of the light period. Controls remained in high R:FR ratio (black bars). b. The percentage change in hypocotyl of controls compared to low R:FR ratio treated seedlings. Hypocotyls were measured 24 h after the end of the treatment. Error bars represent SE. b Shows percentage change in hypocotyl lengths. A sample size of 15 was used for each genotype.

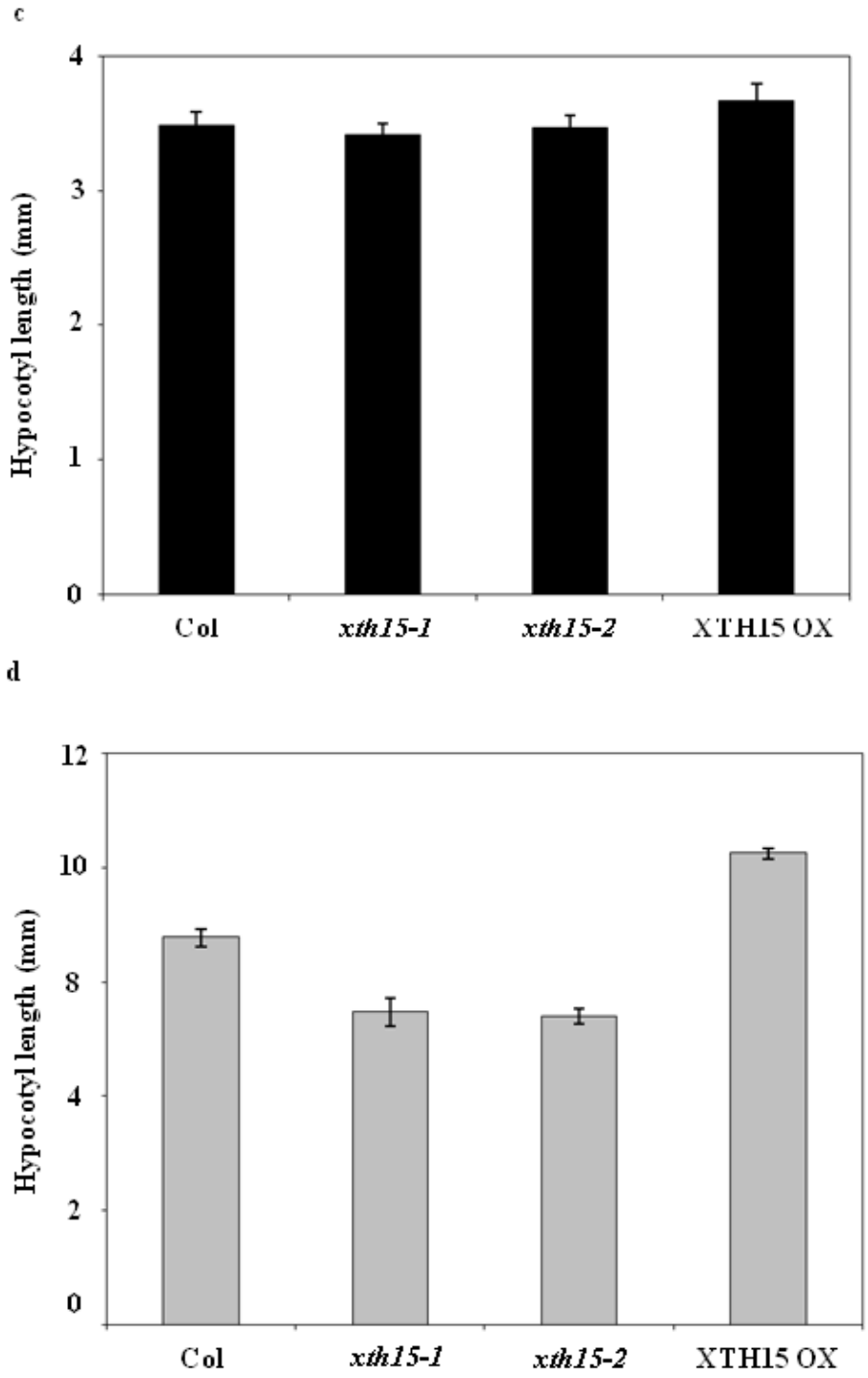


Figure 5.2.8.3. c. Hypocotyl lengths of the relevant wild-types and mutant *xth15* plants grown for 5 days in high R:FR ratio (black bars) 12 h light/ 12 h dark cycles at 22°C. d. Hypocotyl lengths of the relevant wild-types and mutant *xth15* plants grown for 5 days in low R:FR ratio (grey bars) 12 h light/ 12 h dark cycles at 22°C. Hypocotyls were measured 24 h after the end of the treatment. Error bars represent SE. A sample size of 15 was used for each genotype.

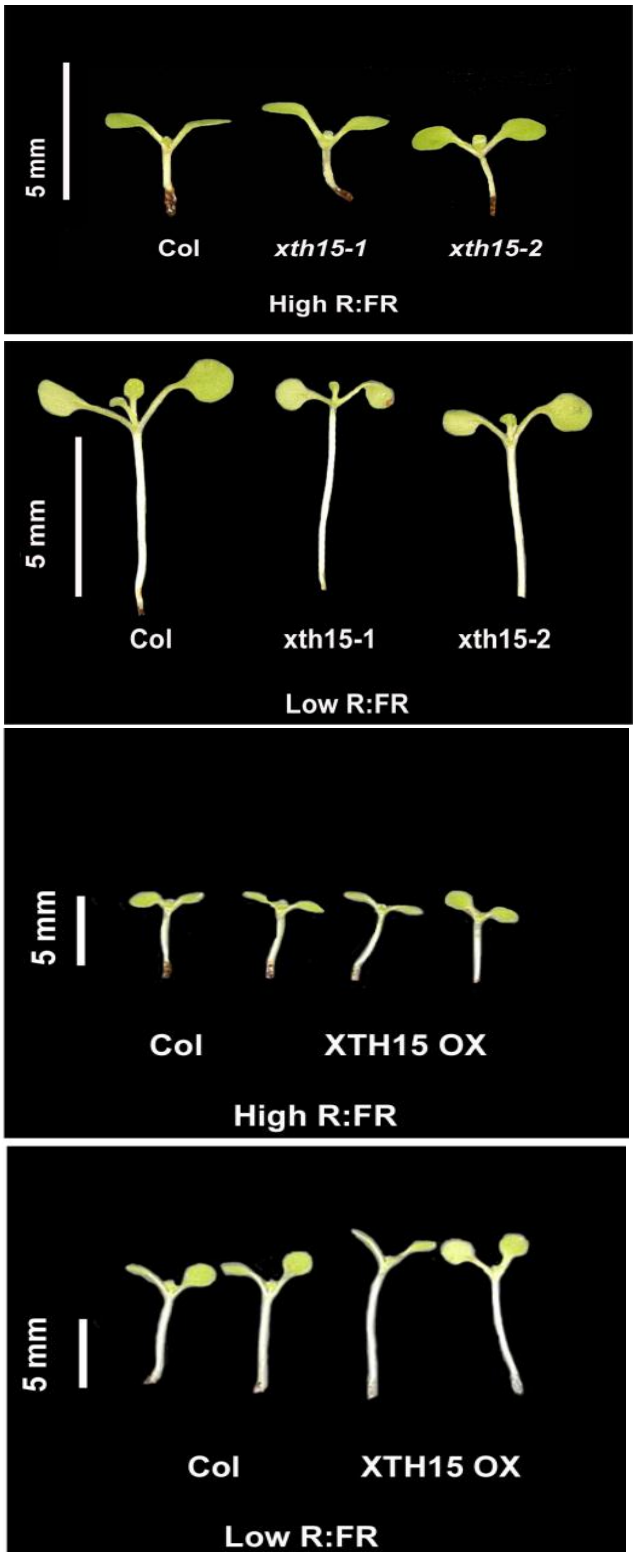


Figure 5.2.8.3. e. The effect of low R:FR ratio and high R:FR ratio on hypocotyl length. The relevant wild-types and mutant *xth15* plants grown for 5 days in either low or high R:FR ratio 12 h light/ 12 h dark cycles at 22°C.

5.2.8.4. Photon irradiance dependency of XTH15 inhibition of hypocotyl elongation

A visible hallmark of photomorphogenic responses is the de-etiolaton of seedlings under specific wavelengths of light. The previous section described how seedlings lacking functional *XTH15* show a small reduction in hypocotyl length following either a transient or prolonged reduction in R:FR ratio. The following section examines the photomorphogenic responses of the *xth15* mutants and XTH15 over-expressers under differing photon irradiance conditions. It is possible that aberrant hypocotyl growth responses in XTH15 transgenic lines could depend upon photon irradiance. Thus, seedlings lacking appropriate levels of *XTH15* may show increased sensitivity to specific photon irradiances of light.

To determine whether *XTH15* plays a role in mediating phytochrome-dependent inhibition of hypocotyl elongation, mutants and transgenic seedlings were grown under various photon irradiances of WL, R and FR light (section 2.2.5.) for 4 days. The seedlings were then removed and the hypocotyls photographed and measured using ImageJ (see section 2.2.6.). The results demonstrate that the *xth15* mutants and XTH15 over-expressers retain a fluence rate dependent response in WL, R and FR light. At lower photon irradiances (1 µmol m^{-2} s^{-1}, 0.1 µmol m^{-2} s^{-1} and 0.01 µmol m^{-2} s^{-1}) in WL (Figures 5.2.8.4.1.), R (Figure 5.2.8.4.2.), light, *xth15* mutants show enhanced inhibition, whereas the XTH15 over-expressers show a reduced inhibition of hypocotyl elongation when compared to wild-type. The mutant response is indicative of hyper-sensitivity to R and WL light whilst interestingly, the XTH15 over-expressers display the reverse: Hypo-sensitivity to the same photon irradiances. At lower photon irradiances (1 µmol m^{-2} s^{-1}, 0.1 µmol m^{-2} s^{-1} and 0.01 µmol m^{-2} s^{-1}) of FR (Figure 5.2.8.4.3.) mutant seedlings show a reduced hypocotyl

elongation response whereas, the XTH15 over-expressers display an exaggerated hypocotyl elongation response.

The difference in hypocotyl lengths between wild-type, *xth15* mutants and XTH15 over-expressers is pronounced under WL. To determine whether this was the result of a B light effect, both lines were grown in B light (experimental procedure as above). Seedlings lacking functional *XTH15* grown in B had a short hypocotyl phenotype when compared to wild-type at high photon irradiances (100 µmol m^{-2} s^{-1}) as well as low photon irradiances (0.01 µmol m^{-2} s^{-1}) (figure 5.2.8.4.4.). This result indicates that *XTH15* may act in the blue light-mediated hypocotyl elongation pathway.

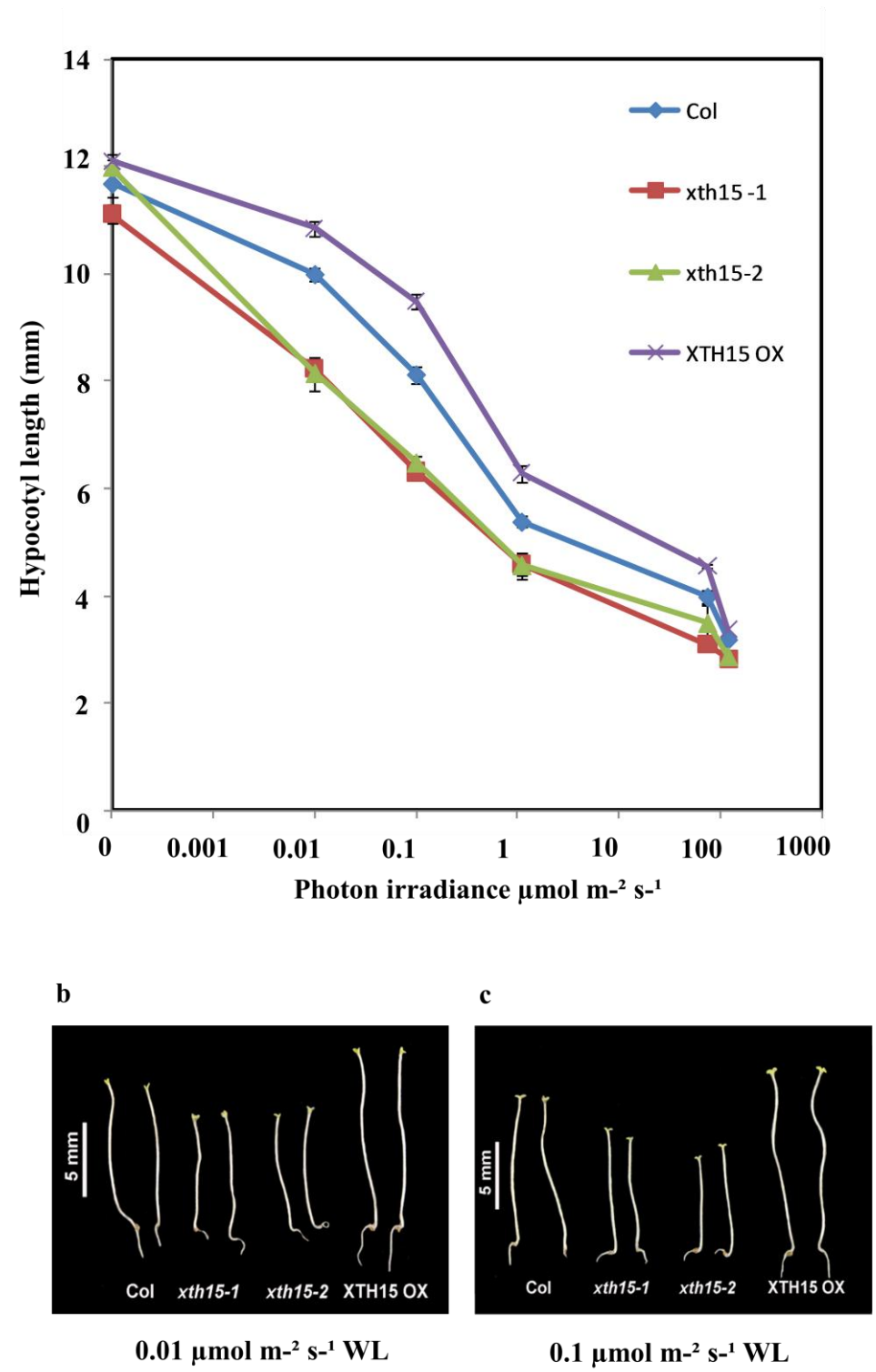


Figure 5.2.8.4.1. a. Hypocotyl lengths of *xth15* mutants, XTH15 over-expressers and the wild-type seedlings grown for 4 days in continuous WL. b. Wild-type, *xth15* mutants and XTH15 over-expressing seedlings grown in continuous 0.01 µmol m-² s-¹ WL for 4 days. C. Wild-type, *xth15* mutants and XTH15 over-expressing seedlings grown in continuous 0.1 µmol m-² s-¹ WL for 4 days.

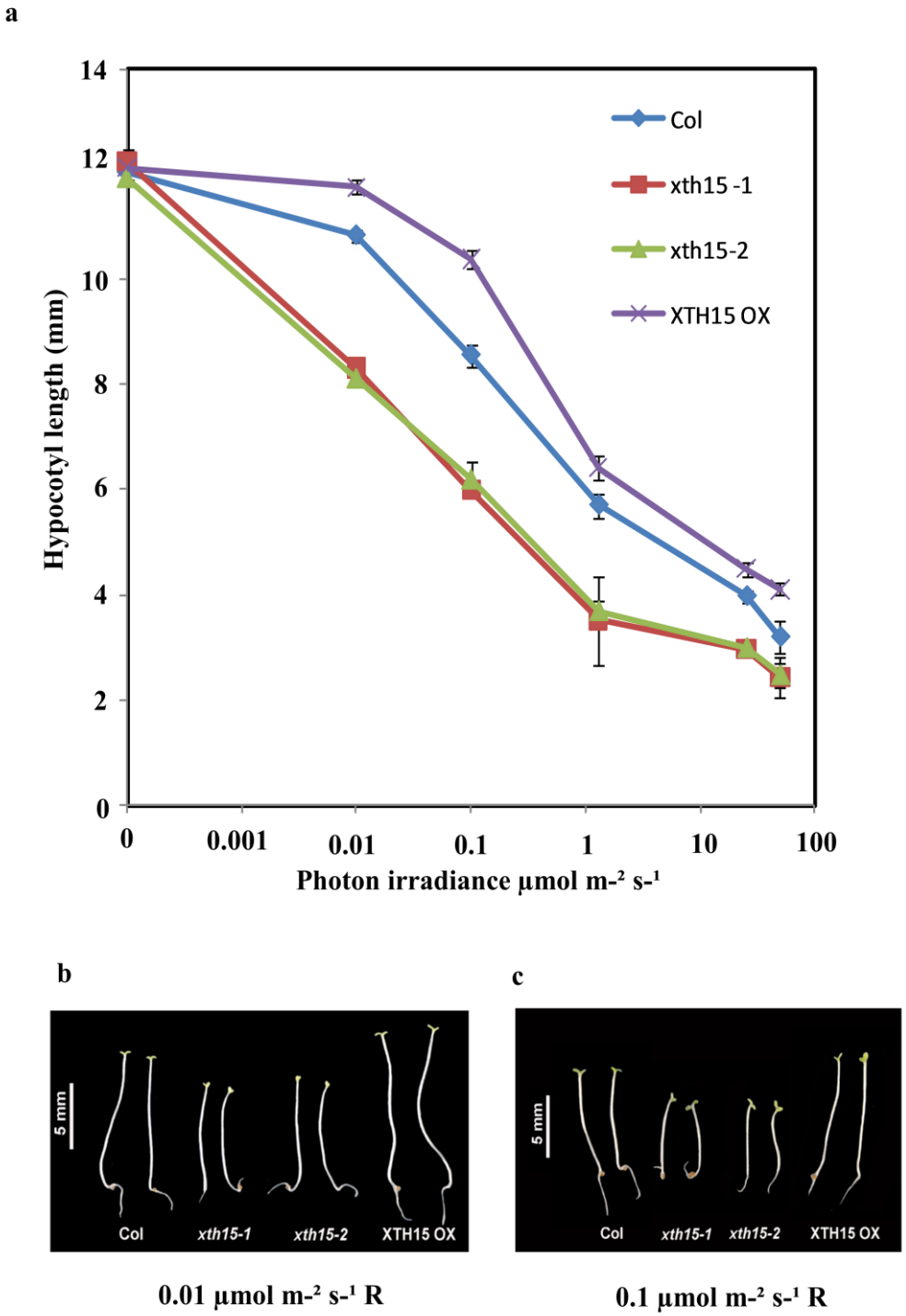


Figure 5.2.8.4.2. a. Hypocotyl lengths of *xth15* mutants, XTH15 over-expressers and the wild-type seedlings grown for 4 days in continuous R. b. Wild-type, *xth15* mutants and XTH15 over-expressing seedlings grown in continuous 0.01 µmol m-² s-¹ R for 4 days. C. Wild-type, *xth15* mutants and XTH15 over-expressing seedlings grown in continuous 0.1 µmol m-² s-¹ R for 4 days.

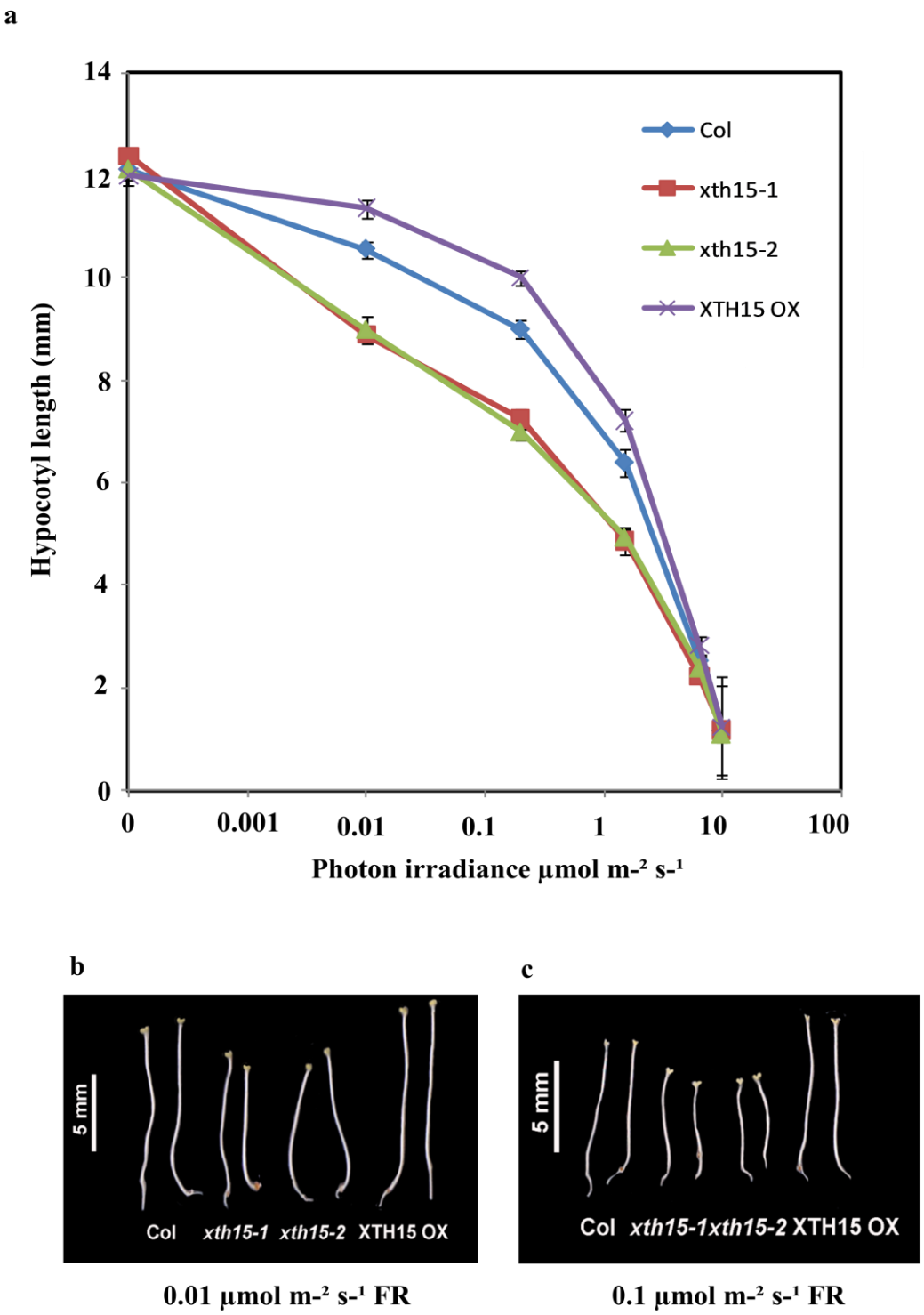


Figure 5.2.8.4.3. a. Hypocotyl lengths of *xth15* mutants, XTH15 over-expressers and the wild-type seedlings grown for 4 days in continuous FR. b. Wild-type, *xth15* mutants and XTH15 over-expressing seedlings grown in continuous 0.01 µmol m-² s-¹ FR for 4 days. C. Wild-type, *xth15* mutants and XTH15 over-expressing seedlings grown in continuous 0.1 µmol m-² s-¹ FR for 4 days.

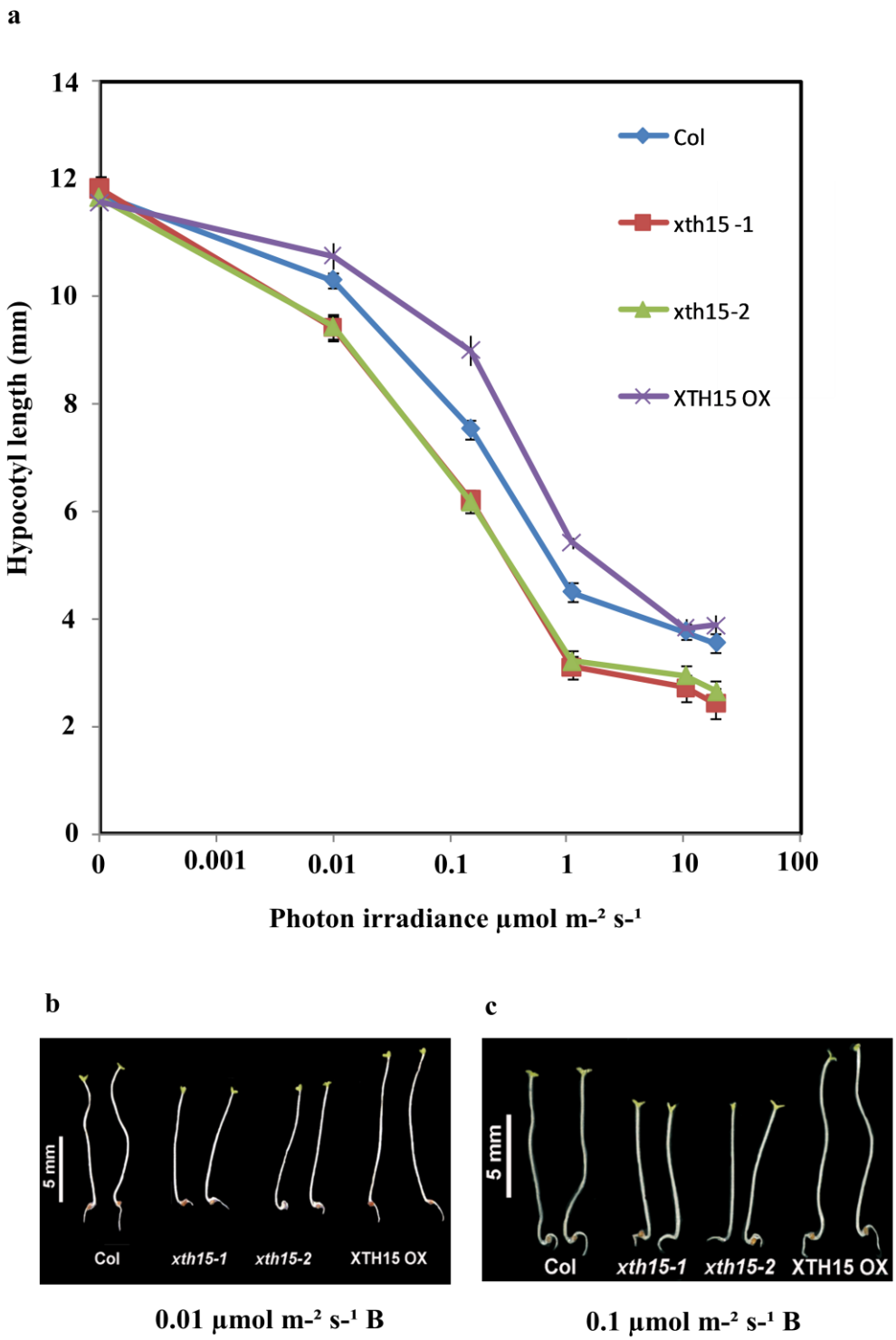


Figure 5.2.8.4.4. a. Hypocotyl lengths of *xth15* mutants, XTH15 over-expressers and the wild-type seedlings grown for 4 days in continuous B. b. Wild-type, *xth15* mutants and XTH15 over-expressing seedlings grown in continuous 0.01 µmol m-² s-¹ B for 4 days. C. Wild-type, *xth15* mutants and XTH15 over-expressing seedlings grown in continuous 0.1 µmol m-² s-¹ B for 4 days.

5.2.8.5. The effect of reduced R:FR ratio on petiole elongation

During periods of low R:FR ratio light, petioles elongate in order to promote greater light capturing capability. Phytochrome B is the photoreceptor that initiates this response. It is also the photoreceptor that has the strongest influence on *XTH15* gene expression. Since petiole elongation occurs via cell wall modifications, XTH15 may be a key component of this process.

To test if this was the case, *xth15* mutants, XTH15 over-expressers and wild-type plants were grown (see section 2.1.3. for details) for 7 days in high R:FR ratio 12 light/ 12 h dark cycles. Subsequently, they were moved to low R:FR ratio light for a further 7 days (section 2.2.4.) whilst controls remained in high R:FR ratio light. The petiole length of leaf 5 was measured (according to section 2.2.6.) from a total of 15 plants from each treatment.

As the results in figure 5.2.8.5. demonstrate, mutants and over-expressing lines show a clear high R:FR ratio light induced increase in petiole length that is indistinguishable from that of wild type plants. However, in low R:FR ratio conditions the *xth15* mutants have shorter petioles when compared to wild-type. In contrast, over-expressers have enhanced petiole elongation when compared to wild-type in low R:FR ratio conditions. In summary, there appears to be a correlation between petiole elongation and XTH15 expression in low R:FR ratio light.

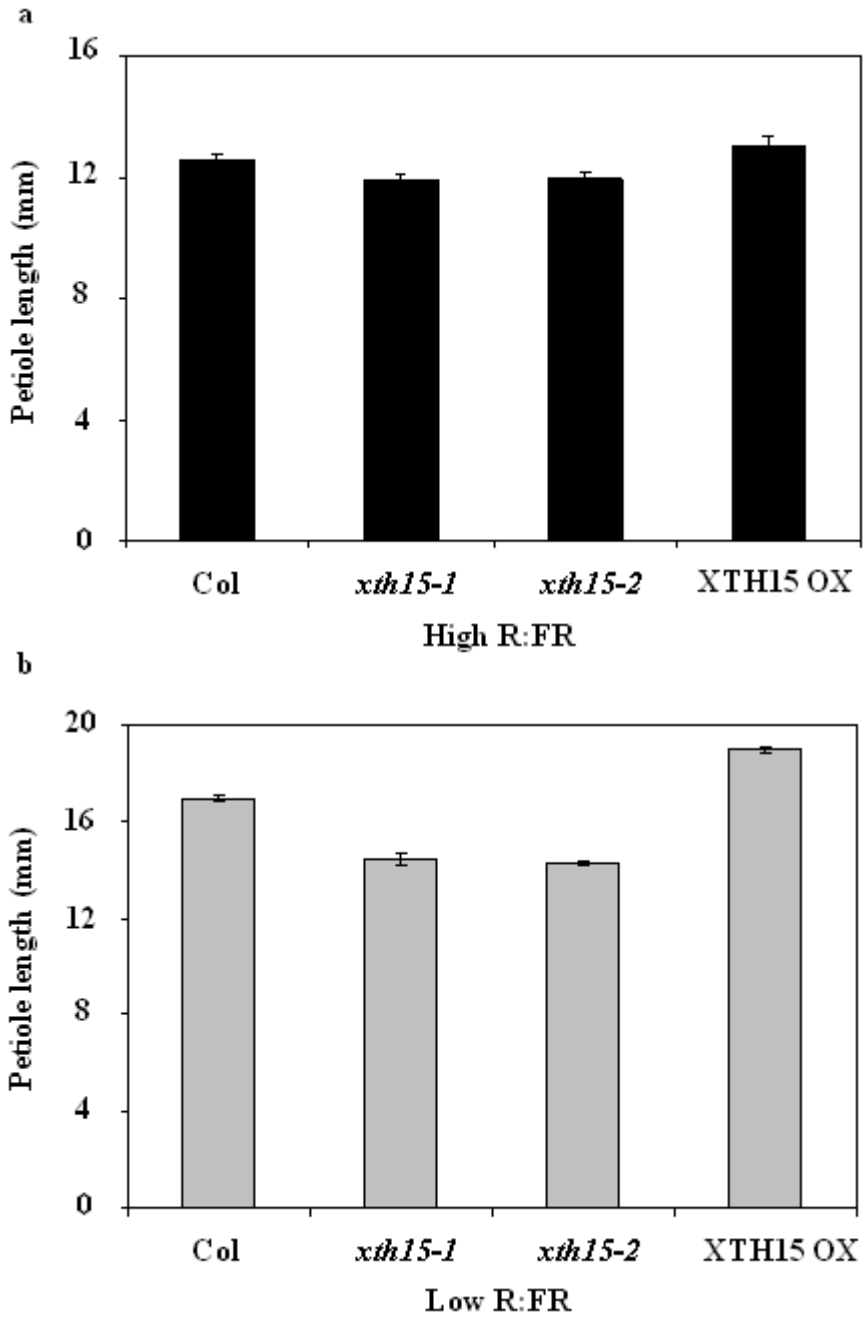


Figure 5.2.8.5. Petiole length of relevant wild-types, mutant *xth15* and XTH15 over-expressing plants, grown for 14 days in high R:FR ratio 12 light/ 12 h dark and then they were moved to low R:FR ratio for a further 7 days (b. grey bars). Controls remained in high R:FR ratio light dark cycles (a. black bars). Petiole length was measured at the end of the 7 day treatment. A sample size of 15 was used for each genotype.

5.2.8.6. The effect of reduced R:FR ratio on flowering time in xth15 mutants and XTH15 over-expressers

A prolonged decrease in the R:FR ratio light environment causes a promotion of flowering time. This response is under the control of phyB, phyD and phyE. The results demonstrate that plants lacking functional *XTH15* have an abnormal petiole and leaf area phenotype when exposed to prolonged low R:FR ratio light treatment. Thus, it is also possible that flowering time is altered in *xth15* mutants under these light conditions.

To identify whether *XTH15* is required for low R:FR ratio induced flowering wild-type plants, *xth15-1*, *xth15-2* mutants and XTH15 over-expressers were germinated (2.1.3.) and then grown in 12 h light / 12 h dark high R:FR ratio for 7 days. The seedlings were then transferred to soil trays (2.2.4.) and either exposed to high R:FR ratio 12 h light / 12 h dark or moved to low R:FR ratio 12 h light/ 12 h dark. Plants remained in these conditions until the onset of bolting. Flowering time in this experiment was determined according to the number of rosette leaves on each individual plant.

Plants subjected to prolonged low R:FR ratio show hastened flowering when compared to plants that remained in high R:FR ratio. The results show (figure 5.2.8.6.a and figure 5.2.8.6.b) that *xth15* mutants and XTH15 over-expressers exhibit a wild-type like flowering response. It is therefore unlikely that *XTH15* plays a role in the acceleration of flowering in response to low R:FR ratio. Figure 5.2.8.6.b shows wild-type, *xth15-1*, *xth15-2* and XTH15 over-expressers at the onset of bolting in both high and low R:FR ratio light conditions.

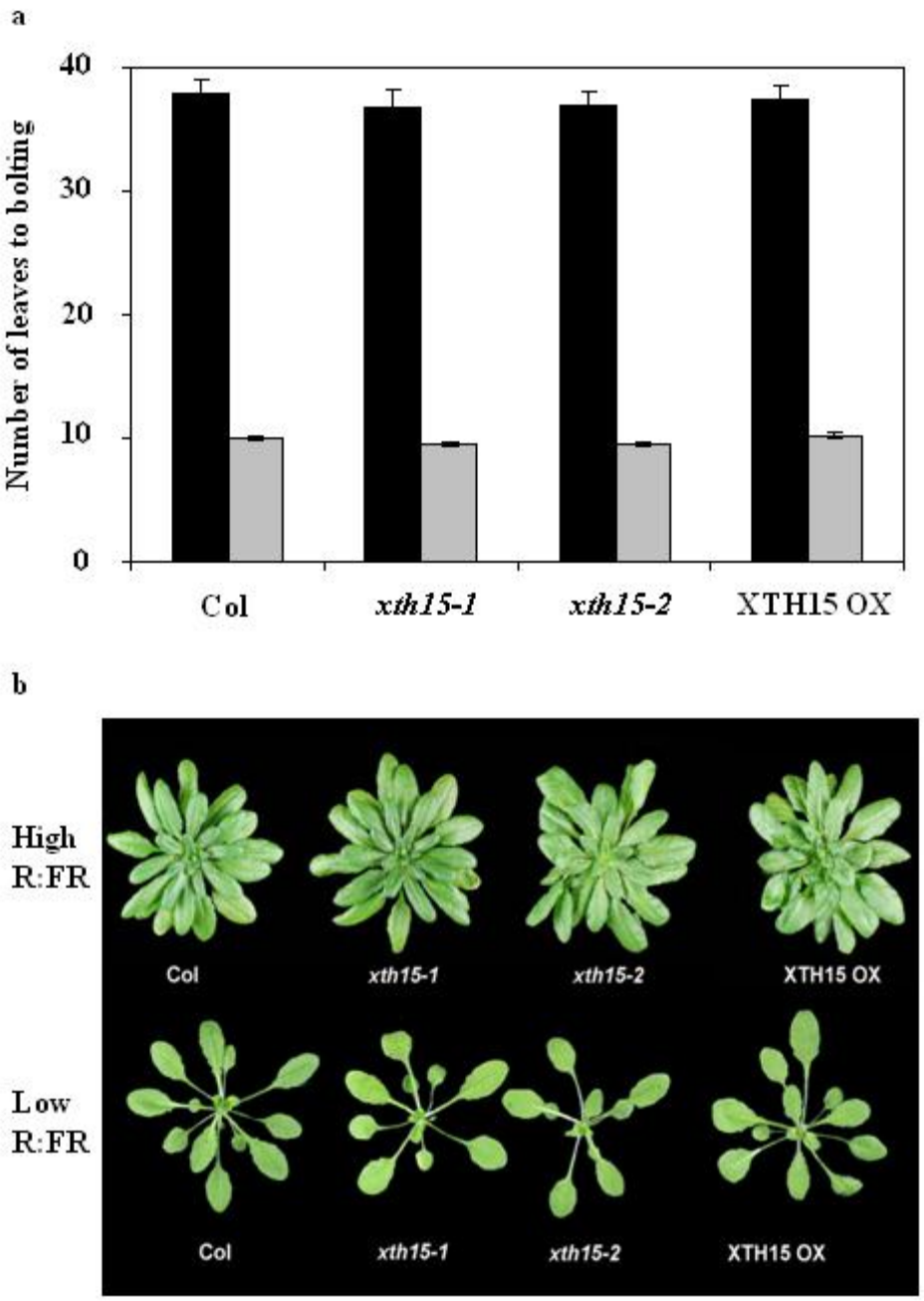


Figure 5.2.8.6. a. Wild-type, *xth15* mutants and XTH15 OX were grown for 7 days 12 h light/ 12 h dark. The seedlings were then moved to 12 h light/ 12 h dark low R:FR ratio conditions or they remained in high R:FR ratio. The number of rosette leaves were then counted at the onset of bolting. A sample size of 15 was used for each genotype. b. Images of plants at bolting in high and low R:FR ratio conditions.

5.2.9. The level of *XTH15* transcript relevant to different photon irradiances of WL

Data presented in section 5.2.8.4. describes exaggerated inhibition of hypocotyl elongation in seedlings grown in WL lacking functional *XTH15* and the converse phenotype in those that over-express this gene. As it is also known that at lower photon irradiances of WL all seedlings have longer hypocotyls than at higher photon irradiances, it is possible that at lower photon irradiances a greater abundance of XTH15 is necessary in order for cell wall modification to occur.

To test this hypothesis, wild-type seedlings were germinated and grown (section 2.1.3.) for 5 days at various different photon irradiances of WL. Tissue samples were then taken for Quantitative RT-PCR analysis (Section 2.5.1.2., 2.5.2.6.). Figure 5.2.9. shows that at 1.5 µmol m^{-2} s^{-1} of WL an 8-fold increase of *XTH15* relative transcript is seen when compared to 150 µmol m^{-2} s^{-1} of WL. This highlights the potential importance of *XTH15* in hypocotyl elongation regulation: At low photon irradiances of WL levels of *XTH15* transcript increase as a reduction in light quantity occurs. This presumably triggers hypocotyl elongation in prolonged shade conditions.

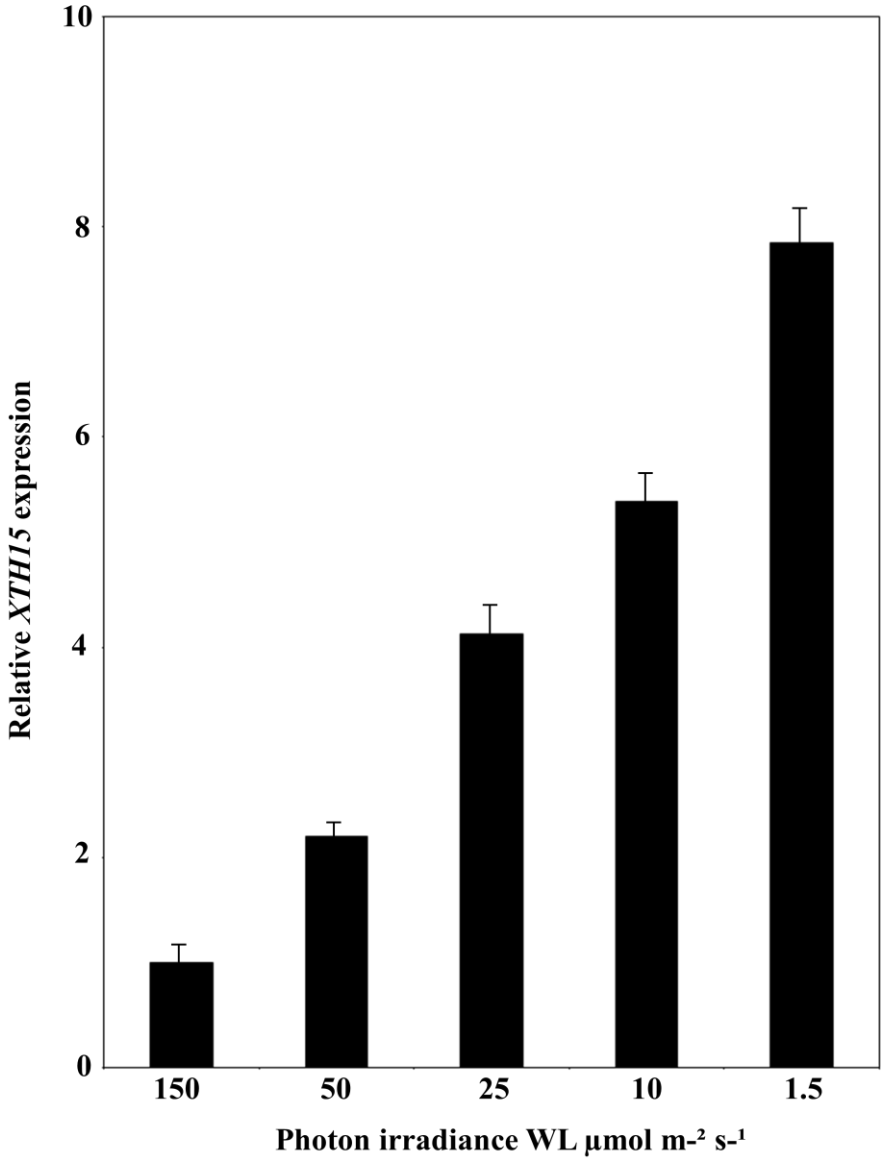


Figure 5.2.9. Transcript abundance of *XTH15* was measured in seedlings grown for 5 days at different photon irradiances of continuous WL at 22ºC using Quantitative RT-PCR. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to the wild-type control.

5.2.10. Cryptochrome regulation of *XTH15*

Screening under B, R and FR light has shown that *xth15* mutants have an over sensitive hypocotyl elongation response (see section 5.2.8.4). Additionally, *XTH15* expression in R and FR light conditions is controlled by phytochromes (see section above 5.2.8.4. and 5.2.3.2.). However, the photoreceptors mediating B light regulation of *XTH15* remain to be determined. Among the potential candidates, cryptochromes (cry) 1 and 2 are potential regulators of *XTH15* expression as they are known to regulate a range of different responses such as de-etiolation (Koornneef *et al*, 1980) and photo-entrainment of the circadian clock (Somers *et al*, 1998).

To assess a possible role for cry1 and cry2 in transcriptional regulation of *XTH15*, *cry1*, *cry2*, *cry1cry2* and the relevant wild-type seedlings were germinated and grown (section 2.1.3.) in high R:FR ratio 12 h light/ 12 h dark photoperiods. On day 5 the seedlings were harvested and Quantitative RT-PCR was carried out to determine relative expression of *XTH15* (Section 2.5.1.2., 2.5.2.6.). The results show (figure 5.2.10.) that in the *cry1* mutant, *XTH15* transcript is reduced when compared to wild-type. More interestingly in seedlings lacking functional *cry2* a 7-fold increase in relative transcript is seen. Expression in the *cry1cry2* double mutant was similar to the *cry2* mutant, suggesting that knockout of both genes does not have an additive effect.

These findings suggest that CRY2 is the principle B light-mediated repressor of *XTH15* transcription. Given this, it is perhaps no surprise that the *cry2* mutant is known to have a long hypocotyl phenotype in low irradiance B and WL. Thus, one might envisage that when *CRY2* is absent, *XTH15* expression is elevated and subsequent hypocotyl elongation occurs though modification of the cell wall. Interestingly, *cry2* mutants also have an enhanced hypocotyl elongation phenotype in

response to low R:FR ratio light (Mas *et al*, 2000). This suggests a point of crosstalk between phytochromes and blue light signalling.

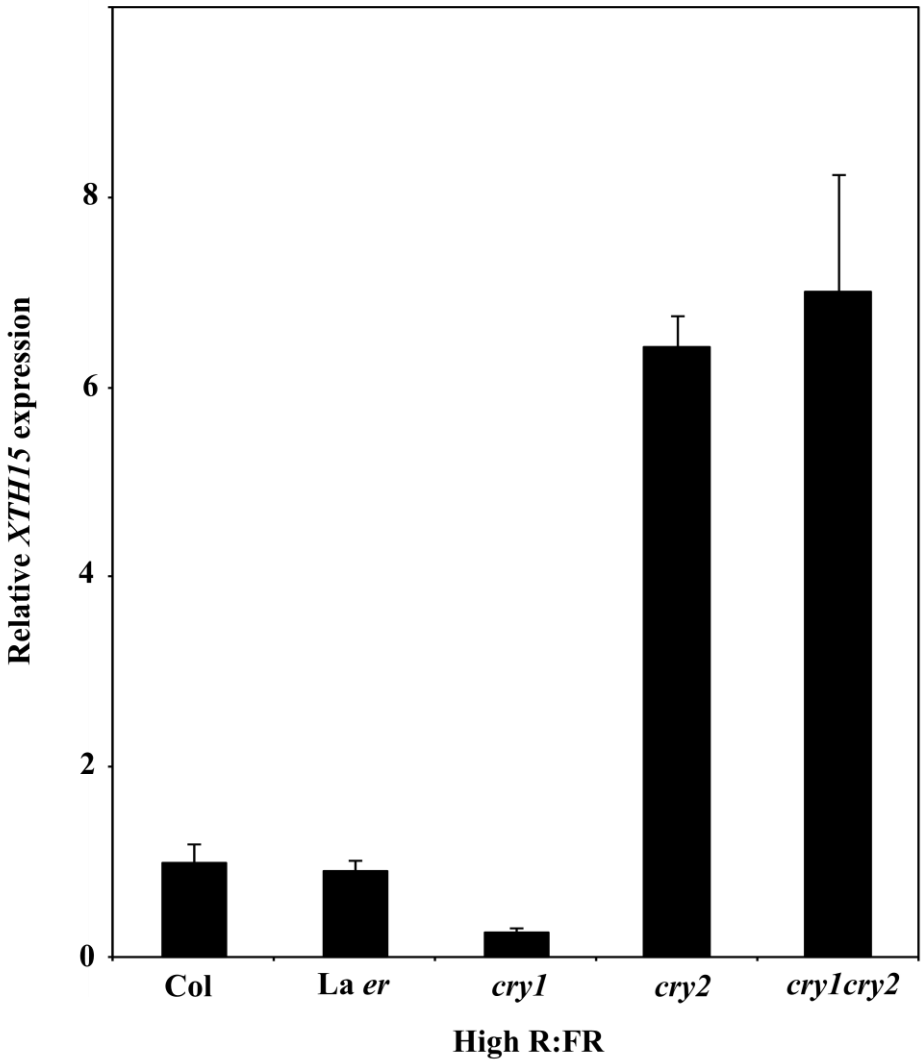


Figure 5.2.10. Cryptochrome-regulation of *XTH15* transcript abundance. Transcript abundance of *XTH15* was measured in 5 day old cryptochrome mutants grown in 12 h light/ 12 h dark cycles at 22°C using Quantitative RT-PCR. Each value is the mean of 3 separate Quantitative RT-PCR reactions normalized to *ACTIN2*. Relative transcript abundance is normalized to the wild-type control.

5.2.11. Identification of promoter motifs

To identify conserved sequence motifs in the promoter region of *XTH15*, a Web Signal Scan Program was used (PLACE). This program identifies previously described motifs within the promoter region. Such analysis was undertaken with the hope that binding domains of known transcriptional regulators of the shade avoidance pathway would be identified: Promoters of light regulated genes are likely to be responsive to the same pathway and therefore share common regulatory motifs. Table 5.2.11. shows all light relevant regulatory motifs in the promoter region of *XTH15*. The results show that *XTH15* contains many light regulatory motifs in its promoter region.

Elements and related motifs	Sequence	Description of motif
-10PEHVPSBD	TATTCT	-10 promoter element found in barley involved light regulation
ATIBOX	AATATTTTTATT	AT-1 in photo-regulation of genes; light regulated genes
CGACGOSAMY3	CGACG	Element found in the GC rich regions. May function as a coupling element for the G box element
GTI Consensus	GRWAAW	Consensus GT-I binding site in many light regulated genes
SORLIP4AT	GTATGATGG	Sequences over represented in light induced promoters
SORLIPSAT	GAGTGAG	Conservation and divergence of light regulated genome expression patterns

Table 5.2.11. Light regulatory motifs found in 1000 bp of *XTH15* promoter region. Promoter elements found using Web Signal Scan Program (PLACE).

5.3 Discussion

The cell walls principle function is to provide a reliable structural enclosure for a plant. However, the cell wall must be capable of dynamic remodelling to accommodate for cell division, differentiation and growth (Carpita and McCann, 2000; Becnel *et al*, 2006). The *XTH* proteins have the enzymatic ability to modify wall xyloglucan polymers by cleaving and joining the newly generated end to another xyloglucan chain or to water, thus allowing wall architecture to be modified. Xyloglucan endotransglucosylases are thought to perform an important role in the regulation and modification of cell wall structure. Since the shade avoidance response causes remodelling of plant morphology, much of which is elongation growth, it is likely that expression of cell wall architecture modifying enzymes, such as *XTH15*, are upregulated when light quality is reduced.

Kinetic experiments confirmed that *XTH15* transcript is indeed rapidly de-repressed in low R:FR ratio conditions, suggesting that *XTH15* is a necessary component of the rapid shade avoidance response. Such rapid changes in gene expression likely enable prompt reshaping of plant morphology, thereby permitting movement towards optimum light conditions. Upon reversion to high R:FR ratio light, *XTH15* transcript falls extremely rapidly indicating that its expression may be controlled by the action of phytochrome isoforms. However, additional regulatory mechanisms also appear to play a role in *XTH15* regulation as prolonged low R:FR ratio light causes a resumption to transcript levels similar to those observed in high R:FR. This suggests negative regulatory mechanisms come into play during extended low R:FR ratio light conditions, possibly acting to reduce the strength of the shade avoidance response which is likely to allow successful long term survival.

The work presented above implicates XTH15 as a key component of the shade avoidance pathway. Thus, it is not surprising that the phyB was found to be the principal phytochrome regulator of *XTH15* expression under changing R:FR ratio light conditions as this is the molecular switch that triggers the majority of physiological responses to changing R:FR conditions. One of these physiological responses is hypocotyl elongation: Seedlings lacking functional *PHYB* are constitutively shade avoiding and therefore exhibit an elongated hypocotyl phenotype. Since XTH15 is an enzyme involved in the remodelling of the plant cell wall, regulation of this gene by phyB may underpin hypocotyl extension during shade avoidance.

Whilst a great many physiological responses to shade are primarily regulated by phyB, they are also subject to moderation by phyA. Therefore, the finding that Phytochrome A represses *XTH15* transcription in low R:FR ratio conditions is perhaps not surprising. The opposing action of phyA on phyB mediated physiological responses is likely to act to ensure that the extent of the shade avoidance response is moderated.

One key component of the shade avoidance pathway is the bHLH transcription factor PIF3. This protein acts a as a bridge between phytochrome activation and transcriptional regulation of shade avoidance genes (Quail, 2002). The work presented in this chapter demonstrates that *XTH15* is part of the signalling cascade targeted by PIF3: Seedlings lacking functional PIF3, have reduced *XTH15* transcript levels in low R:FR ratio conditions. This implicates PIF3 as a positive regulator of *XTH15* expression. The PIF3 protein is a transcription factor that recognises and binds to DNA promoter domains that contain a G-box motif. Since the promoter region of *XTH15* lacks a G-box binding domain, it is unlikely that PIF3 directly interacts with

XTH15. Thus, it is more likely that downstream targets of PIF3 act as transcriptional activators of *XTH15*.

Recent evidence has shown that PIF4 and PIF5 contribute to shade avoidance responses by positively regulating low R:FR ratio responsive genes (Lorraine *et al*, 2007). Hence, *XTH15* could be a component of their signalling pathway. The analysis of *XTH15* expression in *pif4* and *pif5* mutants showed that although transcript abundance increased in low R:FR ratio light, this response was blunted in the *pif4pif5* double mutant. In addition, PIF4 and PIF5 over-expressing lines had substantially greater levels of *XTH15* transcript when compared to wild-type and also had an exaggerated hypocotyl elongation phenotype. Taken together, this evidence points to the control of *XTH15* expression by PIF proteins. Specifically, PIF4 and PIF5 orchestrate the positive regulation of *XTH15* in response to changes in the R:FR ratio light environment. In summary, *XTH15* is one component of a large transcriptional cascade that is triggered by the phytochromes. Several PIF members are upstream elements of this cascade. However, the factors that directly affect *XTH15* expression remain to be elucidated.

There are a total of 33 genes in the XTH gene family. To date, evidence based on sequence similarity and enzymatic function of encoded products indicates that XTH proteins have similar, but not identical function. Thus, one might speculate that members of the XTH family have, at least in part, discrete but overlapping roles in shade avoidance responses. However, despite the large number of genes in this family, the work carried out in this chapter clearly shows that one member, *XTH15*, is necessary for appropriate shade avoidance responses. Thus, at least one member of the family is not redundant. This is substantiated by the single mutant analysis which shows a dramatically perturbed shade avoidance phenotype (figure 5.2.8.2). The

question therefore arises as to whether other XTH members have essential, non redundant roles in the shade avoidance pathway. Unfortunately, due to time constraints, only six members were characterised with regards to R:FR ratio influence on expression. Of these only one (XTH22) showed an altered response to low R:FR ratio treatment. Additional microarray work recently conducted at The University of Leicester found *XTH17* expression to also increase in seedlings exposed to low R:FR ratio light (Allen, pers com, 2007). Interestingly *XTH17* is a phylogenetic member of group two, as is *XTH15*. Thus, it is possible that these two *XTH* genes have overlapping roles, acting in concert to sculpt cell wall properties during shade avoidance.

Whilst the *xth15* mutants retained the ability to undergo hypocotyl elongation in response to low R:FR ratio light, this elongation was reduced, showing marked differences to wild-type responses (figure 5.2.8.2.). Taken together, this suggests that whilst *XTH15* is necessary for appropriate elongation to occur it is clearly not the only cell wall modifying enzyme involved in this response. If *XTH15* does indeed remodel cell walls, then it would be expected that expression occurs in this region of the seedling/ plant. The *XTH15 GUS* fusion analysis revealed that this is indeed the case: GUS expression in these seedlings was observed at the top of the hypocotyl as well as the primary cotelydons regions where the cell wall extension process predominates. The *xth15* mutants also possess a short petiole phenotype in low R:FR ratio conditions (figure 5.2.8.5.). Thus, *XTH15* acts to positively regulate petiole extension in reduced R:FR ratio light. Such a response would be expected to be necessary for permitting elevation of leaves towards unfiltered daylight. Such responses will obviously promote plant survival.

The results of this study strongly support the premise that *XTH15* is a component of the phytochrome-mediated R:FR light signalling pathway, playing an essential part in a number of photomorphogenic responses. Seedlings lacking functional *XTH15* responded hyper-sensitively to R light. Consistent with the mutant phenotype, the over-expression of *XTH15* caused a hypo-sensitive response to R light. It was also found that *xth15* mutants had a reduced response to FR light whilst, over-expression of *XTH15* causes an exaggerated response to FR light. Together, these data suggest that *XTH15* is a component of both phyA and phyB signalling pathways.

The results presented in this chapter also show that XTH15 has an important role in regulating hypocotyl elongation at low photon irradiances of WL. The *xth15* mutants show an increased inhibition of hypocotyl elongation when compared to wild-type at low photon irradiances of WL (1 µmol m^{-2} s^{-1}). In fitting with this observation, XTH15 over-expresser shows the reverse phenotype. Further evidence comes from Quantitative RT-PCR experiments that demonstrate increased *XTH15* transcript levels in wild-type seedlings grown under low photon irradiances of WL (1.5 µmol m^{-2} s^{-1}). This is of particular importance is that *XTH15* appears not only to show significantly increased expression following reductions in R:FR ratio but also following blue light depletion. The *xth15* mutants were found to be hyper-sensitive to B light, a phenomenon that was far more apparent at lower photon irradiances. Regulation of *XTH15* was shown to be mediated predominantly by cry2, with cry1 having little, if any involvement. Interestingly, it has been shown by Mas *et al*, (2000) that *cry2* mutants have elongated hypocotyls when compared to wild-type in low R:FR ratio light conditions. Furthermore, decreasing quantities of B light are known to cause stem elongation in many plant species (Ballaré *et al*, 1991; Casal and Sánchez, 1994). Thus, it is possible that these physiological responses occur, at least

in part, through transcriptional regulation of *XTH15*. When one considers that relatively little is known of the role blue light signalling plays during photomorphogenic shade avoidance responses, the findings presented in this chapter maybe of considerable significance. These data together suggest that *XTH15* is an important component of plants response to shading hence representing a point of convergence with the phytochromes and the cryptochromes.

To conclude, this chapter details the identification of *XTH15* as a novel component of the shade avoidance signalling pathway. This gene is rapidly de-repressed by the actions of phytochrome in low R:FR ratio conditions. Furthermore, its product is ideally suited to act as a modifier of cell walls during shade avoidance: It modifies wall xyloglucan polymers by cleaving and joining the newly generated end to another xyloglucan chain or to water thus allowing the wall architecture to be altered in changing light conditions. This work also identifies *XTH15* as one of the first R:FR ratio regulated genes that is also influenced by B light conditions, thereby possibly representing a point of crosstalk between phytochrome and blue light signalling pathways. Blue light photoreceptors and R/FR phytochromes have been proposed to act synergistically to mediate physiological responses to light. Experiments show that B light responses, such as inhibition of hypocotyl elongation, can be partially reversed by pulses of saturating FR light (Casal, 1994). Such reversibility suggests that phytochrome and blue light signalling pathways could converge and act together to regulate *XTH15*.

6. General Discussion

Plant development is dependent on the ability to perceive and respond to environmental signals. Information that is derived from multiple environmental cues is integrated by immature plants, thereby permitting maximisation of developmental success in a broad spectrum of environmental conditions. Light is one of the most important environmental stimuli influencing plant development as it is essential for ongoing photosynthesis: Successful competition for light is key to developmental success. Angiosperms exhibit highly plastic physiological responses to light stimuli which promote escape from the shade of neighbouring vegetation. When subjected to vegetative shade, exposure to wavelengths of light in the red and far-red frequency range is reduced and this decrease in light quality is detected through phytochrome photoreceptors. This triggers developmental events that result in many morphological changes such as pronounced elongation growth, altered leaf size/ shape and an accelerated onset of flowering. Whilst much is known of the physiological responses that result from light regulation of phytochrome activation, the molecular signalling cascades underpinning these responses have not been fully described.

The identification of multiple R:FR ratio-regulated genes and subsequent mutant analysis has, over recent years, contributed greatly to our understanding of the molecular mechanisms that result in physiological changes observed during shade avoidance. Such molecular dissection of the shade avoidance pathway has revealed considerable complexity within this signalling network. This thesis describes the regulation and possible role of three genes that have previously not been investigated in the context of shade avoidance. These genes putatively define points of interaction between R:FR ratio perception and hypocotyl growth (*XTH15*),

R:FR ratio perception and carbohydrate metabolism (*DIN2*) and a novel component of the R:FR ratio molecular cascade (*ENDO1*). Previous work has suggested important roles for these plants in non-shade avoiding conditions: *DIN2* is thought to promote plant survival under conditions of sugar starvation through β-glycoside conjugate metabolism (chapter 4), XTH15 is predicted to involved in cell wall modifications (chapter 5) and *ENDO1* encodes class IV chitinase, enzymes which have previously been suggested to play a role in plant development (chapter 3). However, little is known of their regulation and function in low R:FR ratio light conditions.

Previous work provided the impetus to study these three genes in detail. Arabidopsis affymetrix oligoarray analysis carried out on plants exposed to a decrease in R:FR ratio identified hundreds of genes which showed marked changes in expression in response to these light conditions (Salter *et al*, 2003). Among these were *DIN2* and *ENDO*, both of which were found to show an elevation of transcript in response to reduced R:FR ratio. A third gene, *XTH15*, was also found to show an increase in transcript in response to low R:FR ratio and was another possible component the shade avoidance response (Franklin, pers com, 2003). This thesis studied the regulation and possible function of all three of these genes in detail, with the hope of unravelling the roles they play during the shade avoidance response. Whilst the affymetrix oligoarray analysis described above confirmed that *ENDO1*, *DIN2* and *XTH15* are regulated by changes in R:FR ratio, little was known of the precise temporal nature of these responses. Through R:FR ratio kinetics experiments carried out in this study, we now understand that *ENDO1*, *DIN2* and *XTH15* show very rapid transcriptional upregulation (time course *ca*. 15-30 min) in response to low R:FR ratio. Such prompt transcriptional regulation of these genes suggests that they are necessary components of the rapid shade avoidance response. It is likely that such

expeditious changes in gene expression are necessary to ensure fast re-shaping of the plant towards a more optimal light environment (Sessa *et al*, 2005).

In addition, experiments carried out during the course of this thesis have found that *ENDO1*, *DIN2* and *XTH15* show classic R:FR ratio reversibility. This study has identified phyB as the predominant phytochrome underlying this reversibility. It is therefore tempting to speculate that *DIN2, ENDO1* and *XTH15* are components of the shade avoiding molecular cascade that are repressed by phyB in high R:FR ratio conditions: When this phytochrome is in its inactive form, these genes are de-repressed and able to exert a developmental influence on the cell wall, allowing elongation growth to occur.

Previous mutant analysis has demonstrated that phyA is necessary for the wild-type shade avoidance response in Arabidopsis (Yanovsky *et al*, 1995; Devlin *et al*, 1996; Whitelam and Devlin, 1997). In fact, it is now widely accepted that phyA acts in an antagonistic manner to phyB. Such a negative feedback mechanism is important for survival: Were it not for the ability of PhyA to curtail excessive elongation growth, exhaustion of resources and premature seedling death would occur in dense vegetation conditions (Yanovsky *et al*, 1995; Smith *et al*, 1997). The findings presented in this thesis demonstrate that phyA acts to decrease the relative expression of *ENDO1*, *DIN2* and *XTH15* in low R:FR ratio conditions. Thus phyA is likely to be acting to prevent disproportionate up-regulation of these genes in dense shade conditions, which could cause detrimental effects to the seedling or plant as a whole. It is worthy of note that the exaggerated elongation responses observed in *phyA* mutants often causes premature death; it is quite possible that the upregulation of cell wall modification proteins such as *XTH15* contribute to this phenomenon (chapter 5).

Thus these genes are repressed by phyB in high R:FR ratio conditions and phyA in low R:FR ratio conditions.

Following photoconversion to the active Pfr form, phytochromes undergo translocation to the nucleus, whereby the nuclear localised Pfr binds directly to bHLH family of transcription factors (PIFs). Molecular and genetic approaches have identified many PIFs are regulated specifically by either phyA or phyB or are common to both (Hudson, 2000; Quail, 2000; Kim *et al*, 2003). This raises the question as to whether *DIN2*, *ENDO1* and *XTH15* expression is also regulated by these key transcription factors. The work presented in this thesis identifies three bHLH transcription factors which control *DIN2* (chapter 4) and *XTH15* (chapter 5) expression during shade avoidance.

The first of these is *PIF3*, a phy-interacting factor that displays photoreversible binding to phyA and phyB Pfr *in vivo* (Ni *et al*, 1998, 1999). In low R:FR conditions PIF3 appears to act to negatively regulate *DIN2* transcription whilst conversely, it positively regulates *XTH15* transcript in low R:FR ratio conditions. PIF3 is known to act as a positive and a negative regulator for phytochrome signalling depending on the light conditions. For example PIF3 positively regulates chalcone synthase whereas, it is a negative component for the inhibition of hypocotyl elongation, cotelydon opening and expansion (Kim *et al*, 2003) The work presented here demonstrates that *DIN2* is negatively, and *XTH15* positively, regulated by PIF3 in low R:FR ratio conditions. Despite this, physiological analysis revealed that plants lacking functional PIF3 do not exhibit an aberrant shade avoidance phenotype in low R:FR ratio. Thus the role of PIF3 in the regulation of these two genes, and the role this regulation has during shade avoidance awaits further clarification. Finally, my work has show that PIF3 must be an indirect regulator of *DIN2* and *XTH15*

expression: PIF3 is known to simultaneously bind to Pfr and a G-Box motif located in the promoter region of several genes (Martínez-García *et al*, 2000). However, as *DIN2* and *XTH15* do not possess G-Box motifs, it is likely that PIF3 is not acting as a direct transcriptional regulator of these genes.

Similar to PIF3, the bHLH transcription factors PIF4 and PIF5 have been shown to interact preferentially with phyB in *vitro*. Recent analysis has shown that PIF4 and PIF5 proteins have increased stability in low R:FR ratio light conditions. In high R:FR ratio it is known that PIF proteins are degraded following binding of photoactivated phytochrome. However, in low R:FR ratio photoconversion occurs and subsequently the nuclear export of phyB Pfr. This results in enhanced stability of PIF4 and PIF5 and allows them to exert a positive influence on shade avoidance gene expression (Lorrain *et al*, 2007). The *pif4* mutant has a short hypocotyl phenotype in both high and low R:FR ratio light and the *pif4pif5* double mutant has a short hypocotyl phenotype along with reduced petiole length in both high and low R:FR ratio light. Both PIF4 and PIF5 over-expressing seedlings have been found to have a constitutively long hypocotyl phenotype however, instability of the PIF4 transgenic lines led Lorrain and colleagues (2007) to focus mainly on PIF5. In conclusion the authors believe that PIF4 and PIF5 promote the expression of genes involved in mediating elongation growth. The work presented in this thesis corroborates this study: Analysis of *pif4*, *pif5* and *pif4pif5* mutants revealed they had reduced expression of *DIN2* and *XTH15* in low R:FR ratio conditions. On the contrary, seedlings over-expressing PIF4 and PIF5 displayed considerably elevated levels of *DIN2* and *XTH15* transcript in high R:FR ratio conditions. It is possible that positive regulation of *DIN2* transcript by PIF4 and PIF5 is a necessary regulatory step which allows DIN2 to utilise β-glycoside conjugates as a carbohydrate source. Such

mobilisation of sugars would permit physiological shade avoidance responses; such as hypocotyl and petiole elongation growth; to occur. Similarly, XTH15 is thought to be involved in the regulation of cell wall architecture through modification of xyloglucan polymers by cleavage and subsequent joining of newly generated ends to other xyloglucan chains or to water. Thus, it is possible that positive regulation of *XTH15* by PIF4 and PIF5 is necessary for the elongation phenotype observed when plants are actively avoiding shade.

Previous studies have revealed that *DIN2* and *ENDO1* are positively regulated by SHL (Mussig and Altmann, 2003). *SHL* is a gene that encodes a small nuclear protein which contains a BAH domain and a PHD finger. SHL is thought to be involved transcriptional regulation and chromatin remodelling factors. Interestingly, plants with reduced *SHL* transcript have a severely stunted phenotype; plants possess fewer leaves, have extremely compact rosettes and flower late (Mussig and Altmann, 2003). Thus, *SHL* appears to be critical for plant growth. In light of this, it is tempting to speculate that in *shl* mutants, genes such as *DIN2* and *ENDO1* are not upregulated and the plant is unable to develop accordingly. Data presented during this study suggests that SHL may be part of the *DIN2* and *ENDO1* transcriptional regulatory pathway and may also be regulated by R:FR ratio. An important step in the future will be to confirm as to whether *SHL* plays any role in the molecular shade avoidance pathway.

The present study identifies PIF3, PIF4 and PIF5 as key transcription factors that regulate expression of *DIN2* and *XTH15,* two genes that are now thought to be part of the shade avoidance pathway. It is now understood that changes in light quality implement a complex transcriptional pathway through the initiation of a master set of transcriptional regulators (such as the PIFs) which result in the rapid

increase in transcript of *DIN2* and *XTH15*. Unfortunately, a high-order regulator of *ENDO1* transcription was not identified in this study. Whilst unfortunate, time restraints and availability of resources (in essence only a handful of the many bHLH transcription factors were analysed) hampered attempts to isolate a regulator of this gene.

On a final note, it is possible that highly related bHLH transcription factors play overlapping regulatory roles in the phytochrome signalling network of which *ENDO1*, *DIN2*, and *XTH15* are three components. It is known however, that PIF3, PIF4 and PIF5 do not act in a completely redundant manner as each single mutant has a characteristic phenotype. Future studies could confirm as to whether this is the case by studying *ENDO1*, *DIN2*, and *XTH15* transcript in plants that harbour genetic lesions in multiple bHLH genes.

The identification of *PIL1* as a R:FR ratio regulated and circadian regulated gene that controls hypocotyl elongation response (Salter *et al*, 2003), generated considerable interest within the field as it opened up the possibility that the circadian clock was involved in control of the molecular machinery underpinning shade avoidance. This work provided the impetus to determine whether the circadian oscillator also has a role in regulation of *DIN2*, *ENDO1* and *XTH15*. The findings presented revealed that low R:FR ratio-mediated *DIN2* (chapter 4) and *ENDO1* (chapter 3) gene expression is indeed under the control of the circadian clock. In contrast, no circadian rhythm can be observed for *DIN2* and *ENDO1* in high R:FR ratio light conditions.

A number of other low R:FR ratio regulated genes were also identified during this study as being clock regulated: *ASC8*, *ATHB2*, *GA20 OX*, and *SULFANOTRANSFERASE* (Data not shown). A number of these were also found to

show circadian regulation of transcript in high R:FR ratio light conditions (*ASC8*, *ATHB2*, and *SULFANOTRANSFERASE*), although the amplitude of these responses was small in comparison to low R:FR ratio light treatment conditions.

The majority of genes identified detailed in this thesis tend to show peak expression 2-4 h after the onset of subjective dawn. This finding seemed paradoxical at the time as it had previously been shown that maximum hypocotyl elongation occurred at dusk (Salter *et al*, 2003). As such, one would expect maximal gene expression fall in register with this time period. However, discoveries made since submission of this thesis clearly shown that under more natural conditions, when plants are exposed to light/ dark cycles, maximum elongation growth does indeed occur at dawn. It is at this time that positive regulators of shade avoidance response (such as PIF4 and PIF5) are most stable due to the presence of high *PIF4* and *PIF5* transcript abundance and enhanced protein stability under these conditions (Lorrain *et al*, 2007). Salter *et al* (2003) exposed plants to continuous light conditions during experiments, which appears to have caused anomalies in R:FR regulated gene expression patterns during their experiments.

The large number of R:FR ratio regulated genes that have also been found to be circadian regulated has lead to the suggestion that all molecular components of the shade avoidance response are regulated in this manner. However this does not appear to be the case. Notably, this thesis demonstrates that *XTH15* is a rapidly R:FR ratio regulated gene that is not controlled by the circadian oscillator in either low or high R:FR ratio light conditions (chapter 5). This is of particular interest as work carried out in chapter 5 established a link between low R:FR ratio induced *XTH15* expression and hypocotyl cell wall modification, yet *XTH15* transcript shows no circadian oscillation. Whilst elongation responses occur at dawn, *XTH15* expression does not

show a peak at this time. Indeed low R:FR ratio light causes *XTH15* transcript to remain elevated for 72 h. This puzzling phenomenon awaits suitable explanation. However, it is possible that *XTH15* is one of many genes controlling hypocotyl elongation. Thus, for this physiological change to take place, other genes would presumably need to be upregulated above a certain threshold before elongation growth could occur. If this is the case, it is possible that many of the suite of genes required for elongation growth are circadian regulated, whilst others (such as *XTH15*) are not. Thus, the circadian clock could still exert an influence on this response, even though some molecular components are not directly affected by it.

All three genes analysed throughout this study have one common feature; they are robustly regulated by changes in R:FR ratio light. However, all three genes are likely to have discrete biological functions. The gene *DIN2* belongs to the β-glucosidase family; enzymes which are thought to utilise cell wall polysaccharides as alternate carbon sources under sugar depleted conditions (Fujiki *et al*, 2001). Whilst previous studies have found *DIN2* to be induced by darkness and repressed by exogenous sucrose (Fujiki *et al*, 2001), nothing was known of its role in shade avoidance. This study has shown that *DIN2* is rapidly and reversibly regulated by R:FR ratio and that this response can be partially repressed by exogenous sugars (chapter 4). Thus, it is possible that the work presented has uncovered a link between low R:FR ratio light environment and free sugar depletion. This is an interesting prospect as it raises the possibility that when plants are subjected to low R:FR ratio light, *DIN2* transcript increases rapidly resulting in the utilisation of ß-glycoside conjugates as carbohydrate sources which subsequently releases sufficient energy for plants to elongate. However, it is important to note that an obvious shade avoidance phenotype was not identified in *din2* mutants during this study. Again it is likely that

other sugar mobilising enzymes act in concert to release free glucose from ß-glycoside conjugates to provide sufficient energy during shade avoidance. To examine this hypothesis further it would be necessary to analyse the effects of disrupting the dark-inducible/ β-glucosidase genes individually and in combination, thereby permitting the study of molecular events that occur during low R:FR ratio/ sugar depleted conditions.

ENDO1 (chapter 3) is known to be regulated by a range of abiotic stressors, such as water deficit (Bray, 2004), high salinity (Takahashi *et al*, 2004) and oxidative stress (Takahashi *et al*, 2004). It is possible that this gene has overlapping functions during a number of stress conditions and shade avoidance. Alternately, the shade itself could simply be an abiotic stress, thereby inducing expression of an identical suite of genes. Thus, it could be possible that low R:FR ratio induces changes in expression of many stress responsive genes.

As loss of *ENDO1* results in aberrant petiole elongation in response to low R:FR ratio, this gene is likely to play a role in the shade avoidance response. As member of the chitinase-like family of proteins, ENDO1 shows homology to Cowpea yieldins, proteins that are known to regulate cell wall growth (Okamoto-Nakazawa *et al*, 2000a; Okamoto-Nakazawa *et al*, 2000b). It is therefore possible that in reduced R:FR ratio conditions, *ENDO1* expression is necessary for cell wall restructuring events during petiole elongation growth. However, whilst *endo1* mutants did show a retarded petiole elongation response to low R:FR ratio when compared to wild-type, petiole elongation was not abolished completely. This suggests that the gene *ENDO1* acts alongside other cell wall modifiers to permit restructuring of the cell wall in reduced R:FR ratio environments. *ENDO1* may also be a stress responsive gene that is up-regulated in response to a number of environmental stressors.

This work has also identified *XTH15* (chapter 5) as an important enzyme involved in regulation and modification of cell wall structure, specifically during low R:FR ratio conditions. The shade avoidance response causes remodelling of plant morphology, much of which is through elongation growth. Through the analysis of *xth15* mutants a biological function has been successfully assigned to XTH15 (chapter 5). Although the *xth15* mutants retained the ability to undergo hypocotyl elongation their response was retarded when compared to wild-type. In addition, *XTH15::GUS* transgenic fusion analysis identified XTH15 accumulation at the top of the hypocotyl. The work presented in this thesis provides evidence that *XTH15* is involved in both the R and FR light regulated molecular pathway. Seedlings lacking functional *XTH15* responded hyper-sensitively to R and hypo-sensitively to FR light, conversely over-expression of XTH15 caused a hypo-sensitive response to R and a hyper-sensitive response to FR light. Collectively, *XTH15* is controlled by both phyB and phyA molecular signalling cascade. Plants lacking *XTH15* were also found to show impaired petiole extension, this is another important elongation response that is necessary for plants to avoid shade successfully.

It is intriguing that *XTH15* plays an important role in hypocotyl elongation at low photon irradiances of light. This suggests that this gene is an important molecular component of a plants ability to respond to actual shading; when plants are shaded by neighbouring vegetation they experience a change in light quality (reduced R:FR ratio) and a reduction in light quantity in particular R and B wavebands. Generally speaking, the role of B light in shade avoidance has remained largely uncharacterised at the molecular level. This study shows the existence of both unique R:FR ratio signalling pathways (*ENDO1* and *DIN2*) and shared R:FR ratio and B light signalling pathways (*XTH15*). Significantly increased expression of *XTH15* occurs in response

to reduced R:FR ratio and B light depletion. The premise that elements within both pathways overlap is supported by previous work demonstrating that a number proteins, such as HY5 (Ang *et al*, 1998) and SUB1 (Guo *et al*, 2001), may play a role in both the phytochrome and cryptochrome signalling pathways. The *xth15* mutants were found to show hyper-sensitivity to B light, an effect which was significantly more apparent at lower photon irradiances. It is plausible that *XTH15* acts to modulate cell wall extension in both the phytochrome and the cryptochrome pathway. Interestingly, *XTH15* is regulated by cry2. Mutations in the *cry2* gene have been reported to respond in an exaggerated manner to low R:FR ratio (Mas *et al*, 2000). It is therefore possible that transcription factors activated by both B and low R:FR ratio light signals act on the same promoter elements of *XTH15* to regulate elongation growth. Whether regulation converges at a common promoter region or at unique promoter motifs within the gene remains to be discovered.

Whilst many low R:FR ratio regulated genes are gated by the circadian clock, *XTH15* does not show such gating. However, this does not exclude the possibility that *XTH15* expression is gated by changes in B light quality. The bHLH transcription factors PIF4 and PIF5 are known to stabilise in conditions of reduced low R:FR ratio (Lorrain *et al*, 2007). Transgenic plants over-expressing PIF4 and PIF5 show considerably elevated levels of *XTH15* transcript and also display an elongated phenotype which is consistent with the shade avoidance syndrome. Taken together, a speculative model for *XTH15* regulation could occur through putative stabilization of PIF4 and PIF5 in B light depleted conditions. Thus, these proteins could act to regulate *XTH15* in both low R:FR ratio light and B light. The results reported in chapter 5, suggest the possibility that other R:FR ratio regulated genes are also regulated by B light signals. Microarrays carried out in the future should enable the

identification of genes that respond to both reduced R:FR ratio and reduced B light. Such further analysis will significantly enhance our understanding of how phytochrome signalling pathways interlink with B light signalling pathways during Arabidopsis development.

6.1. Concluding remarks

Over recent years, molecular biologists have begun to unravel the complex signalling pathways that belie shade avoidance behaviour. The number of shade avoidance components identified has risen exponentially year by year. However, the identification of new genes involved in this process will undoubtedly contribute to our knowledge of how shade avoidance is controlled in higher plants.

As discussed throughout this chapter there are many challenges confronting researchers in the field: the identification of novel shade avoidance components, elucidation of how the signalling pathway acts upon downstream shade avoidance targets, the characterization of mutants harbouring knockouts of genes regulated during shade-avoidance. Notwithstanding these challenges, one major hurdle that researchers face is to determine how shade avoidance is influenced by both R:FR ratio and B light signals. In order to further understand shade avoidance regulation, microarray analysis and promoter motif studies through promotion deletion series may initially be the best path for investigation.

6.2. Future work

6.2.1. Future work: ENDO1

Although it is clear that *ENDO1* is rapidly and robustly regulated by changes in R:FR ratio, this study has not clearly identified the light regulated transcription factor (s)

that exert this effect. Future work is therefore needed to further dissect the transcription factor pathways involved in *ENDO1* regulation. In this context, it would be valuable to analyse *ENDO1* transcriptional responses to both high and low R:FR ratio light in other PIF family mutants, such as *PIF4* and *PIF5*.

Whilst data presented in this thesis suggests that *ENDO1* contributes to petiole elongation responses during shade avoidance, it is possible that it has additional functions that were not uncovered during the course of this study. The screening of plants which over-express *ENDO1* under the control of the CaMV 35s promoter, in comparison with null mutants will provide more information concerning the role of *ENDO1*. Further physiological characterisation of *endo1-1*, *endo1-2* and ENDO1 over-expresser would possibly allow other biological roles to be assigned.

In addition, multiple knockout lines would be a valuable tool for assessing further functions of chitinase-like genes during shade avoidance. Of the nine chitinase genes in Arabidopsis only five are thought to produce functionally active products. Combinatorial knockout of these five genes would permit determination of the role this family of genes plays during shade avoidance. However, it should be noted that of these five genes only *ENDO1* is transcriptionally upregulated in low R:FR ratio conditions (see Chapter 3).

6.2.2. Future work: DIN2

Data suggests that *DIN2* responds to multiple signals including darkness, changes in R:FR ratio and cellular sugar levels. Preliminary data suggests that a decrease in R:FR ratio alters the availability of carbohydrates within the cell (Whitelam pers com 2006). It would therefore be extremely interesting to compare the availability of free sugars in *din2* mutants and *DIN2* over-expressers. This would provide important

additional evidence that *DIN2* is involved in utilization of β-glycoside conjugates as a carbohydrate source during shade avoidance.

Determining the spatiotemporal expression pattern of DIN2 may provide pointers towards its potential biological role. It is possible for instance that expression is maximal in areas of the seedling/ plant that undergo elongating. To investigate expression patterns, northern blotting and reporter gene analysis could be implemented. It would also be possible to determine the localisation of DIN2 within the cell using YFP fusion analysis. Screening of seedlings which over-express *DIN2* under the control of the CaMV 35s promoter, in comparison with null mutants, will provide more information concerning the role of DIN2.

Further physiological characterisation of *din2-1*, *din2-2* mutants and DIN2 over-expresser would provide valuable information concerning the role of *DIN2*. Of particular interest is the possible interaction of *DIN2* with the axillary bud out growth pathway. It is possible that known axillary pathway genes could show alterations in transcript levels in *din2* mutants and in DIN2 over-expressers. By analysing a number of axillary branching genes (e.g. *max1* and *max2*) by Quantitative RT-PCR in *din2* mutants and *DIN2* over-expressers when compared to wild-type would allow such aberrant responses to be identified.

In order to understand whether β-glucosidase genes are required for appropriate shade avoidance responses it would be valuable to determine which of the 48 β-glucosidase-like gene family members are transcriptionally regulated in low R:FR ratio conditions. My work has shown that *DIN2* is upregulated in these conditions whereas *DIN6* and *DIN9* are not. This leaves 45 β-glucosidase genes that have not yet been examined in the context of shade avoidance, although eight of these can be excluded on the assumption that they are pseudogenes. Quantitative RT-PCR

of plants exposed to high and low R:FR conditions would allow one to determine precisely which β-glucosidase genes are regulated during shade. Once this has been determined multiple knockouts could be generated to allow determination of a biological function for β-glucosidases during shade avoidance. Obviously if a large number of these genes are upregulated, knocking them all out would present a major challenge.

6.2.3. Future work: XTH15

All the data collected to date points to a role for XTH15 in cell wall architecture modification. To confirm such a sub-cellular expression pattern reporter gene analysis can be implemented. The localisation of XTH15 could be determined by YFP fusion analysis. The screening of seedlings, which over-express XTH15 under the control of the CaMV 35S promoter in comparison with null mutants, would provide more information concerning the regions where XTH15 is active.

The recent findings that *XTH15* has a role in regulating hypocotyl elongation at low photon irradiances of WL and B light suggests that *XTH15* is an important component of plant responses to actual shading, therefore representing a point of cross over between phytochrome and B light signalling. It is possible that R:FR ratio signals and B light signals act through a similar or unique promoter motif. Determining such promoter regions would allow us to predict other R:FR ratio and B light regulated genes. This could be carried out by the construction of transgenic plants expressing *XTH15* promoter deletion series fused to *LUC*. Expression of LUC could be quantified following R:FR ratio and B light reduction treatments.

In order to determine whether plant responses to reductions in R:FR ratio and B light are either additive or synergistic; a physiological marker such as hypocotyl

length could be measured in response to individual and combined light treatments. Hypocotyl length could be compared in R light, R light plus B light, B light plus FR light and R light plus FR light. The PAR will remain the same in all the different light treatments. To investigate the relative expression of XTH15 in the relevant light treatments Quantitative RT-PCR could be carried out following the transfer of the seedlings to the light conditions.

To study the regulation of *XTH15* gene expression in high and low R:FR ratio, reporter gene fusions using *GUS* were analysed in seedlings and expression was found to be mainly located at the top of the hypocotyl. However, *XTH15* expression patterns have not been analysed at any other stages of development. It would therefore be interesting to analyse the role of XTH15 in adult plants grown in both high and low R:FR ratio. The *XTH15::GUS* reporter fusion could allow a putative role to be assigned to *XTH15* in various adult stages of development. It has now been established that B light also plays a role in *XTH15* regulation and an analysis could be carried by analysing the reporter fusion in plants grown in various light conditions.

A physiological function has been identified for XTH15 as seedlings lacking are defective in hypocotyl elongation under low R:FR ratio conditions. Carrying out analysis of cross sectioned hypocotyl tissue in mutant and wild-type seedlings grown in high and low R:FR ratio may allow the cellular basis of the growth defect to be found. It is also possible that cellular defects may be found in other parts of the seedling therefore, cross sections from other parts of the plant may result in greater understanding of *XTH15* and its ability to modify cell wall structure.

6.3 Addendum

6.3.1. DELLAs; mediators of the GA pathway

Giberellic acid (GA) is a phytohormone that controls several important aspects of plant growth such as seed germination, hypocotyl/ stem elongation, and flowering. Expression of this hormone is controlled by light: GA is expressed at high levels in the hypocotyls of dark grown seedlings, whereas those grown in light display significantly lower GA expression. A number of recent studies have identified enzymes that act as key regulators of GA expression under different light conditions, notably, GA20-oxidase and GA3-oxidase, which promote GA synthesis and GA2-oxidase, which renders the protein inactive. Archard *et al*, (2007) found that there are high levels of GA20-oxidase mRNA and low levels of GA2-oxidase in dark grown seedlings and conversely there are high levels of GA2-oxidase mRNA and low levels of GA20-oxidase in light grown seedlings.

GA and phytochrome mediated pathways may interlink to regulate elongation growth. A key component point of convergence may be a family of growth repressing proteins called the DELLAs of which there are five members: RGA, GA1, RGL1 RGL2 and RGL3 (Feng *et al*, 2008). Studies have shown that in dark grown seedlings there are high levels of GA, which act to negatively regulate the DELLA proteins. In contrast, when seedlings are grown in light, GA levels are very low DELLA proteins accumulate. Seedlings grown on a medium with supplemented GA have notably longer hypocotyls than controls. However, if seedlings are grown on a medium containing GA inhibitors, they have shorter hypocotyls than the controls. Thus it is believed that DELLA proteins act to restrain growth whilst GA promotes growth by overcoming the actions of DELLA. This allows a mechanism to be in place whereby

light, via GA and the DELLA protein signalling, can control hypocotyl length. The mechanism underpinning GA-mediated control of DELLA expression has recently been identified: The DELLAs bind to the GA receptor GID1 (Ueguchi- Tanaka *et al*, 2005; Griffiths *et al*, 2006; Najajima *et al*, 2006; Willeige *et al*, 2007). This complex subsequently interacts with the F-box protein SLEEPY1 (SLY1) resulting in polyubiquitination and degradation of the DELLAs via the 26S proteasome (Mcginnis *et al*, 2003).

6.3.2. Interaction of DELLAs and PIFS

The DELLA proteins regulate several genes that respond to GA. However, chromatin immunoprecipitation (chIP) experiments have so far failed to identify direct association between DELLA proteins and GA responsive genes, therefore suggesting that the DELLAs indirectly regulate expression of these genes (Feng *et al*, 2008).

One proposed mechanism is that the DELLAs, rather than directly regulating GA responsive gene expression, may regulate GA responsive genes through the PIFs. This has recently been confirmed by yeast two hybrid screens and in vitro pull downs which demonstrate direct PIF3 interaction with each of the 5 DELLA proteins (Feng *et al*, 2008). chIP and PCR techniques determined that an increase in the amount of nuclear localized DELLAs results in the proteins interacting with PIF3, thereby preventing PIF3 from binding to promoters of target genes. The DELLAs therefore abrogate PIF3 mediated light control of hypocotyl elongation. In contrast, when GA levels are increased, PIF3 is able to bind to its target genes and regulate hypocotyl growth (Feng *et al*, 2008). Therefore PIF3 and DELLAs function antagonistically with respect to the expression of PIF3 target genes.

Immunoprecipitation assays have shown that the DELLAs also interact with PIF4 proteins. Further deletion studies found that PIF4 RGA interact via the bHLH DNA recognition domain in PIF4 and a conserved leucine repeat motif in the RGA protein (De Lucas *et al*, 2008). chIP studies show that this physical interaction blocks transcriptional activity thereby inhibiting PIF function. Thus whilst it is well know that phyB negatively regulates PIF4 expression, it also appears that the DELLAs similarly affect this protein (De lucus *et al*, 2008).

Recent studies indicate a positive influence of both PIF4 and PIF5 factors on expression of cell elongation genes such as *XTH15,* thereby allowing elongation growth in response to low R:FR ratio conditions. In high R:FR ratio conditions it is known that phyB negatively regulates PIF4 transcription by targeted degradation and that the DELLAs repress transcriptional activity of PIF4 by interacting with the bHLH DNA recognition domain and sequestering these factors into an inactive complex. Therefore, stabilisation of the DELLA proteins represses PIF4 mediated elongation growth. However, GA acts to destabilise the DELLAs allowing free PIF4 in the nucleus and the subsequent activation of PIF4 regulated elongation genes (De lucas *et al*, 2008). In *Arabidopsis* RGA and GA1 are the main repressors of hypocotyl growth and stem elongation (Din *et al*, 2001; Peng *et al*, 1997). The above findings of the DELLA/ PIF interaction provide insight into how R:FR ratio and GA converge to regulate elongation growth.

6.3.3. Low R:FR ratio mediated regulation of DELLAs and GA

It has now been shown that DELLAs are degraded more rapidly in low R:FR ratio conditions, confirming the involvement of phytochromes in their regulation. Similar degradation was also observed in B light depleted conditions, suggesting that DELLA

proteins are regulated by multiple photoreceptors (Djakovic Petrovic *et al*, 2007). The hormone GA is known to be involved in hypocotyl and petiole growth responses. This has been confirmed through the study of GA deficient mutants. Mutants deficient in GA display short hypocotyls in response to low R:FR ratio and B light depletion. Quadruple mutants deficient in the DELLAs (*gai*/ *rag*/ *ragl1*/ *rgl2*) have an elongated hypocotyl phenotype but they still remain responsive to low R:FR ratio and B light depletion. This suggests that low R:FR ratio mediated DELLA degradation is not sufficient to induce a shade avoidance response but along with multiple signalling mechanisms performs a more central role in hypocotyl elongation (Djakovic Petrovic *et al*, 2007). A further role for GA has been found in prolonged shade conditions as there is an increase in the expression of transcripts encoding GA 20-oxidase which is known to be important in GA synthesis (Sessa *et al*, 2005). It is therefore possible that in prolonged low R:FR ratio conditions there is an increase in GA synthesis leading to a decrease in DELLA stability and subsequently an increase in free PIF4 binding to its target elongation genes.

With the recent advances in the field concerning the interaction between light, GA, DELLA proteins and the PIFs, it is likely that the regulation of GA signalling by DELLA proteins and the DELLA/ PIF interactions are key in the regulation of known PIF regulated genes such as *DIN2* and *XTH15*. Such findings advance considerably our understanding of how R:FR ratio mediated signalling and GA pathways converge to regulate elongation growth. It is perceivable that *XTH15* is one of the necessary elongation genes located at the end of this molecular cascade. It is possible that as the DELLAs are more rapidly degraded in both low R:FR ratio light and B light depleted conditions (Djakovic Petrovic *et al*, 2007) that they are regulated by both the

phytochromes and the cryptochromes. Therefore, DELLAs could be key players in the regulation of *XTH15* both low R:FR and B light.

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Appendix 1. Primers used in this study

Primers for Salk line testing (LBb1 5'-GCGTGGACCGCTTGCTGCAACT-3')

endo1-1 and *endo1-2*	F 5'- ACCGATTTGCCCTTTTCTTC-3'
	R 5'- TGCGAACATTAGTGGTCCAG-3'
din2-1	F 5'- AACACCTAAGTGAGAACCCGC-3'
	R 5'-:ACACATTTGAAATCGTAGCCG-3'
din2-2	F 5'- TCTTGGTTTTGTACCGTCGTC-3'
	R 5'- ATCATTCCGATTCAACTGACG-3'
xth15-1	F 5'- GGTGACCACAGAGGCAAAAT-3'
	R 5'- AATCCACGAGGGAACCTTTT-3'

Primers used for RT-PCR

ENDO1 Product = 700bp	F 5'- CCTGATCCTAACCGTTTCCA-3'
	R 5'- AACTCCAAGCTTGCCACAAT -3'
DIN2 Product = 650bp	F 5'- CCGATTTTCAATCTCGGGT-3'
	R 5'- GCTCGAACCATCTTGGTGAT-3'
XTH15 Product = 490bp	F 5'- GGTGACCACAGAGGCAAAAT-3'
	R 5'- GAGCCTTGGACCAGTCAGTC-3'

Primers used for over-expresser lines

XTH15 OX	F 5'- GCCTCGAGATGGGTCCAAGTTCGAGCCTCACCATCG-3'
	R 5'- CGACTAGTTCAGACTCTGGACTTCTTGCTTCTGGAGG-3'
DIN2 OX	
	F 5'- CGGATCCATGGCTAAGGGATCGTGGTTCTTCATTATCC-3'
	R 5'- CGGGTACCTCAATAAAATGAAGATGGGTTCTCTATGC-3'

Primers used for 35S::ENDO1::YFP

35S::ENDO1::YFP	F 5'- GCCTCGAGATGGCTAAACCCACATCACGAAATGACCG-3'
	R 5'- CGGGTACCACAAGTGAGGTTATCTCCAGGTGC-3'

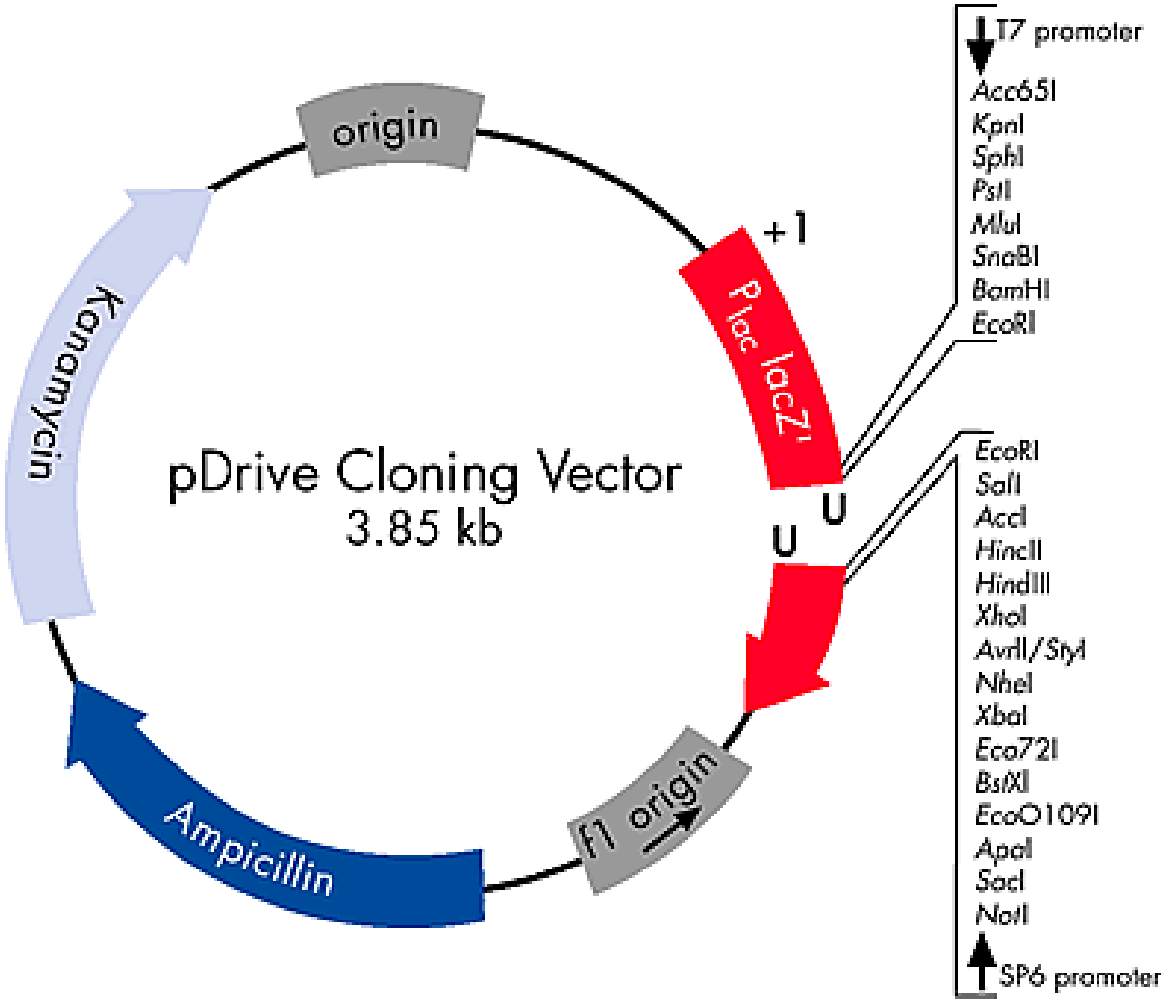
Quantitative RT-PCR Primers

ENDO1	F 5'-TAGCTTCGGTGCTTCCATCT-3'

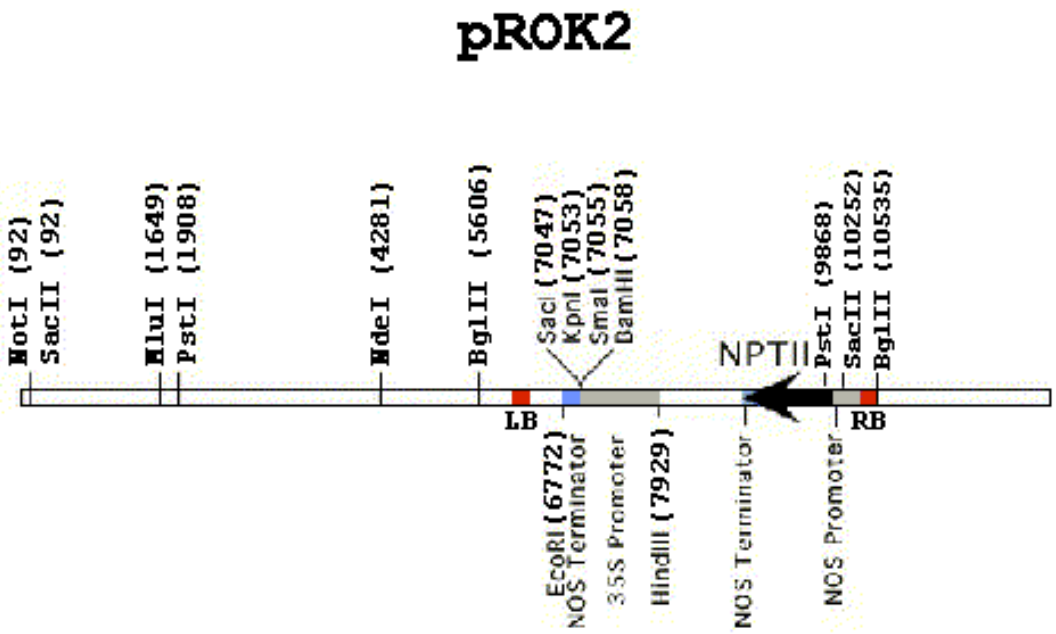
	R 5’- GCACATGGGAACTCTGGTTT-3’
DIN2	F 5’- TGGAAGATGGGTGTGACGTA-3’
	R 5’- CTCCGACGACCGATTTCTTA-3’
XTH15	F 5-1:TGGCGACTGTTCTTCTTGTG-3’
	R 5’- TCCTCCGTTGAAGATTTTGC-3’
At2g43590	F 5’-:CCCAACTTTGCCAATTCTGT-3’
	R 5’- ATTGTGTGTTGCTGCTCTGG-3’
At2g43610	F 5-1:TCGCAGCCTACAAAGGAACT-3’
	R 5’- ATGTTGTGCTTGGTGAGCAG-3’
At2g43620	F 5-1:GTGCAGCTGGAAAGTTCCTC-3’
	R 5’- AACCACATGGCACACTGAAA-3’
At3g54420	F 5-1:TGAGATTGCAGCGTTCTTTG-3’
	R 5’- GTTGCATTCTCGTCGCAGTA-3’
DIN6	F 5-1:TCAACGCCTTATGAGCCTCT-3’
	R 5’- GGCAACAAGGGAAGAATCA-3’
DIN9	F 5-1:TTCAAGCCCTTTGTGGTTTC-3’
	R 5’- TCTCGTCTTCATCGTGTTCG-3’
XTH3	F 5-1:GTCTCTGGAACGTGACGAT-3’
	R 5’- TCTCGGAAATGTGCAATGAA-3’
XTH4	F 5-1:TTCCCATTCAACCAACAAT-3’
	R 5’- TGGCAACCATCTATGTGGAA-3’
XTH5	F 5-1:GCAACGAATCAACCTCTGGT-3’
	R 5’- CACCCCAACGTCTTTGCTAT-3’
XTH16	F 5-1:ACTTGGGGTGAACACAGAGG-3’
	R 5’- AGTTACCGGCGACGAGTTTA-3’
XTH22	F 5-1:CTACTGGCTCGTGGTTGTCA-3’
	R 5’- CCTCTTCGCATCCGTACAAT-3’
XTH24	F 5-1:CAATCCTCTGGAACCCTCAA-3’
	R 5’- ACATCCTCATCGGCTTGTTC-3’

Appendix 2.

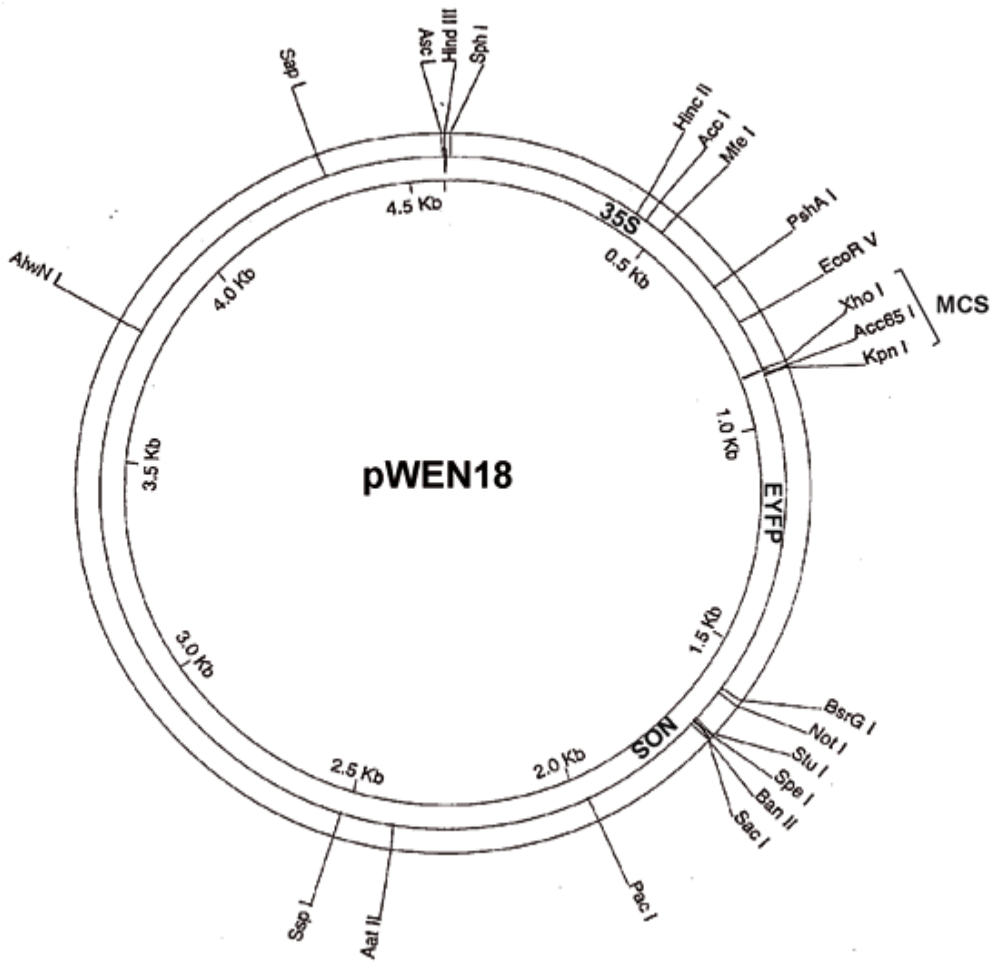
Vectors used in this study:



Appendix 2. 1. pDrive (Invitrogen)



Appendix 2.2. pROK2 (SALK)



Appendix 2.3. pWEN18 (Kost et al., 1998).