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LIAM EDWARD O'HARA

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**Liam Edward O'Hara**

**The identification of potential cis- and trans-acting factors in the regulation of *DARK INDUCIBLE 3 (DIN3)* expression during darkness and chilling in *Arabidopsis thaliana***

**Abstract**

Plant responses to environmental stimuli are co-ordinated by a variety of sensing and signalling mechanisms, which bring appropriate internal changes so that plants are able to adapt to a changing environment. It was the aim of this project to investigate the regulation of one gene: *DARK INDUCIBLE 3 (DIN3)*, specifically the cis- and trans-acting factors. To achieve these aims, the investigative approach centred on gene expression analysis of linker-scan mutation analysis of 50 base-pairs (bp) of the minimal functional promoter of *DIN3*. To investigate the contribution made by trans-acting factors, the effects of over-expression of candidate transcription factor genes were analysed. This project determined that in addition to dark-induced expression already described in the literature, the dark-induction of *DIN3* expression could be repressed by low temperature. Specific motifs within the crucial 50bp of the *DIN3* promoter were found to be necessary for dark-induced expression, which together was hypothesised to constitute a sugar-responsive sequence. No cis-acting regulatory motifs were found to contribute definitely to the cold-responsiveness of *DIN3*. None of the transcription factor genes investigated, were revealed to have a major role in the dark and cold responsiveness of *DIN3*. The results of this project suggest that there is considerable cross-talk between dark/sugar regulation and low temperature at the cis- and trans-acting level.

**The identification of potential cis- and trans-acting factors in  
the regulation of *DARK INDUCIBLE 3 (DIN3)* expression  
during darkness and chilling in *Arabidopsis thaliana***

**Liam Edward O'Hara B.Sc.**

**Submitted for the degree of Master of Science by research**

**Department Biological and Biomedical Sciences**

**University of Durham**

**2010**

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## **Dedication**

The author wishes to dedicate this work corporately to the Plant Stress and Signalling Lab at the University of Durham, in which it was a pleasure and a privilege to work. May it be granted every success in the future.

## **Introduction**

This project concerns the contribution of various cis- and trans-acting regulatory factors of *DARK INDUCIBLE 3 (DIN3)* during chilling and darkness. In order to provide a better account of the research undertaken the current understanding light, temperature and sugar sensing and responses has been reviewed and is presented here below, starting with light sensing then proceeding to consider temperature and sugar signalling, finally exploring plant responses to low light conditions

### **1.1.1 Light Sensing**

Light is, for plants the most dominant environmental signal that can be perceived, unsurprising given their autotrophic nature. Light is also the dominant factor influencing plant growth and development. This is not a new understanding, Charles Darwin made detailed observations of the dramatic transformation that seedlings undergo when they are exposed to light for the first time (photomorphogenesis), as well as growth towards the direction of light (phototropism) (Franklin & Quail, 2009). Dark-grown (etiolated) and light-grown (de-etiolated) seedlings have strikingly different morphologies, the former has highly elongated hypocotyls, closed (maintained apical hook) and unexpanded cotyledons and an absence or low abundance of photosynthetic pigments in etioplasts. By contrast, seedlings that have been de-etiolated have reduced hypocotyl elongation, apical hook opening, expansion of the cotyledons and the synthesis of photosynthetic pigments in plastids (Franklin & Quail, 2009; Terzaghi & Cashmore, 1995). Plants are able to perceive the quantity (fluence), quality (wavelength) periodicity (day length) and direction of the light that they receive (Franklin et al., 2005). This ability allows plants to co-ordinate their growth and development appropriately for the prevailing environmental conditions. Light perception information is mediated by a group of proteins known as photoreceptors. The photoreceptors can be divided into three families: the phytochromes, which absorb red (R) / far-red (FR) light and the cryptochromes and phototropins, which both absorb blue / ultraviolet-A (UV-A) (Franklin et al., 2005).

#### ***Phytochromes (Red and Far-red light perception)***

The first conclusive evidence for the role of phytochromes in plant development was provided by Borthwick and colleagues (Borthwick et al., 1952) who demonstrated that germination of lettuce seeds was dependent on exposure to minute levels of red light (R) (Very Low Fluence Response – VLFR) and inhibited by FR even after R treatment.

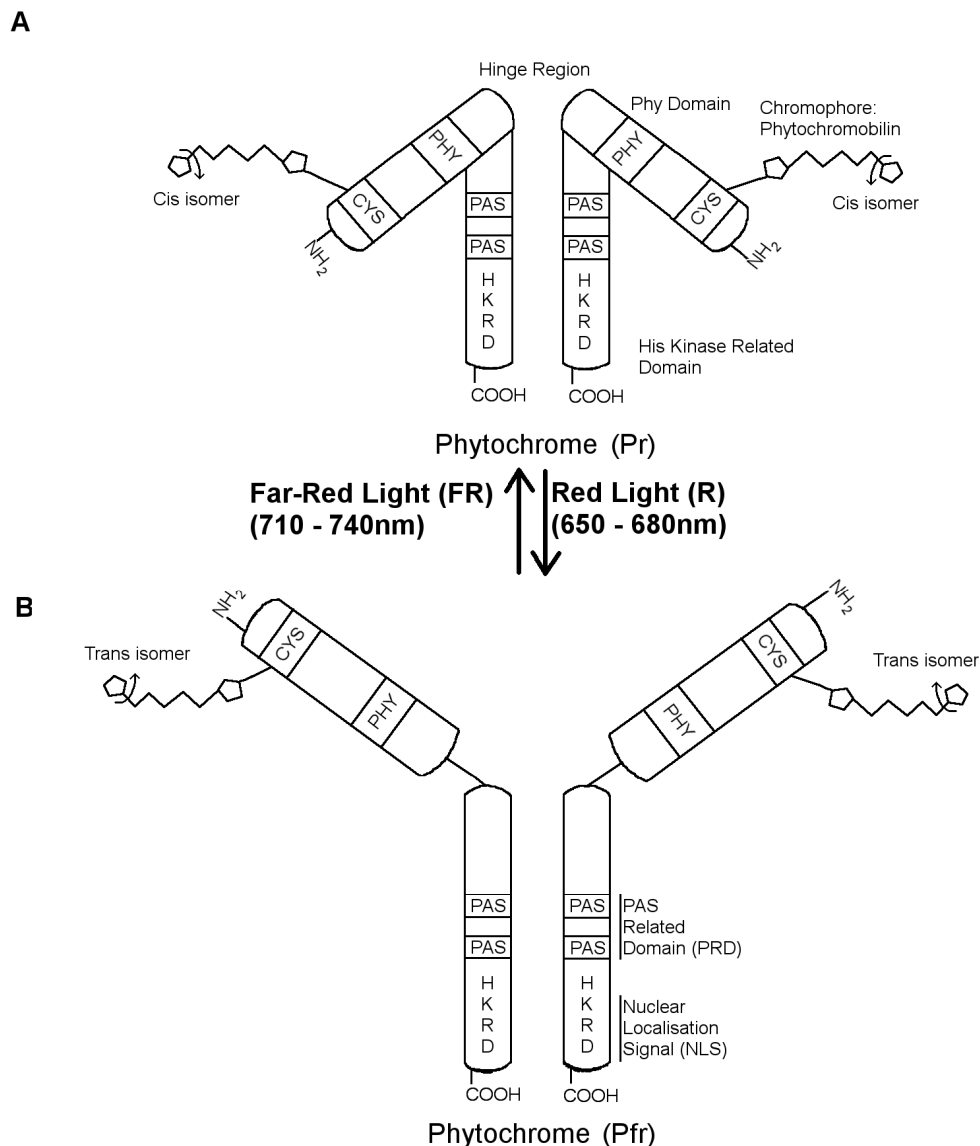
They proposed the presence of a single photoreceptor (phytochrome) that was reversibly inter-convertible between two forms through the action of R and FR (photo-reversibility). In their model phytochrome existed in an inactive Pr, R sensitive form, and an active Pfr, FR sensitive form. Accumulation of sufficient Pfr, through the conversion of Pr by R, would trigger the onset of germination (Franklin & Quail, 2009). *In vitro* confirmation of the inter-convertibility of a single phytochrome from Pr to Pfr, was achieved by (Butler et al., 1959).

### ***Phytochrome structure and function***

Phytochrome is understood to be a soluble ~125kDa chromoprotein (holoprotein – complex of an apoprotein and a chromophore) forming a ~240kDa homodimer (Schäfer & Bowler, 2002) each consisting of two functional domains located towards the opposite ends of the molecule (fig. 1.1.1.1) (Nakasako et al., 2005). The 70kDa photosensory domain is located at the N-terminal end of the molecule and the 55kDa regulatory domain on the C-terminal portion. The photosensory domain includes a cysteine residue to which is attached a linear tetrapyrrole chromophore – phytochromobilin, together known as the bilin lyase domain (BLD). A part of the BLD exhibits  $\alpha$ -helical structure following the conversion from Pr to Pfr, this structure stabilises the Pfr form to facilitate the functioning of the molecule. Towards the proximal end of the N-terminal portion, closer to the regulatory domain, there is the phytochrome (PHY) domain. The PHY domain gives the molecule its absorption spectra specificity. The regulatory domain proper is composed of two period and single-minded, and the vertebrate aryl hydrocarbon receptor nuclear transporter (Per/ARNT/Sim) PAS-related domains (PRDs) within which reside an essential signal transduction motif called a Quail box (QB). Located on the proximal half of the N-terminal portion and one histidine-kinase-related domain (HKRD) – a histidine paralogue with Ser/Thr kinase specificity. The HKRD is overlapped by a nuclear localisation signal (NLS), which facilitates the translocation of Pfr to the nucleus (Nakasako et al., 2005).

Phytochrome apoproteins exist in three conserved forms across the angiosperms (phyA – C), a further two (phyD & E) are present in *Arabidopsis thaliana*. phyA (formerly type I phytochrome) is the dominant phytochrome found in etiolated seedlings and is rapidly converted from Pr to Pfr in a VLFR, thereby allowing swift de-etiolation. Under higher R fluence phyA is degraded and found at much lower levels. However under FR high irradiance, where the Pr form is dominant, phyA undergoes rapid photo-cycling

between the forms, which produces the signal. Phytochromes B – E (formerly type II) mediate the responses to low fluence (LFRs) and high irradiance (R-HIR) in red light, where the Pfr form predominant and active. This ability of phytochromes to convert between the Pr and Pfr forms is termed photo-reversibility – a dynamic equilibrium is established whereby the Pr or Pfr is favoured. phyB is the most abundant of the phytochromes in de-etiolated plants, which demonstrate LFRs and R-HIRs (Franklin & Quail, 2009; Kreslavski et al., 2009).



**Figure 1.1.1.1 Schematic of a phytochrome molecule illustrating photo-reversibility/conversion**

Phytochromes exist in two photo-convertible forms: an inactive Pr, sensitive to red light (R), and an active Pfr, sensitive to far-red light (FR) (Taiz & Zeiger, 2006). Phytochromes are homodimers consisting of two functional subunits: the photosensory domain on the N-terminal portion and the regulatory domain on the C-terminal portion. Attached to a conserved cysteine residue at the distal end of photosensory domain is a linear tetrapyrrole chromophore – phytylphytyl, known as the bilin-lyase domain (BLD). Proximally, is found the phytochrome (PHY) domain that maintains the spectral absorption specificity. On the regulatory domain there are two PAS-related domains (PRDs) within which reside an essential signal transduction motif

called a Quail box (QB). Distal to the PRDs is the histidine-kinase-related domain (HKRD) a histidine kinase paralogue with Ser/Thr specificity. This region is overlapped by a nuclear localisation signal (NLS) motif, required for nuclear trafficking of the active Pfr form (Nakasako et al., 2005). Adapted from figures found in (Taiz & Zeiger, 2006; Nakasako et al., 2005).

The conformational change from Pr to Pfr (fig. 1.1.1.1) is associated with a change in the localisation of the phytochromes. Using green fluorescent protein (GFP) (Yamaguchi et al., 1999) and immunological analysis (Hisada et al., 2000), R irradiation causes some of the Pfr pool to be trafficked from the cytosol to the nucleus, a process reversible with FR treatment (fig. 1.1.1.2). Once in the nucleus, tagged Pfr molecules aggregate into what might be a transcriptome complex to bring about changes in gene expression (Nagy & Schäfer, 2000; Quail, 2002; Kreslavski et al., 2009).

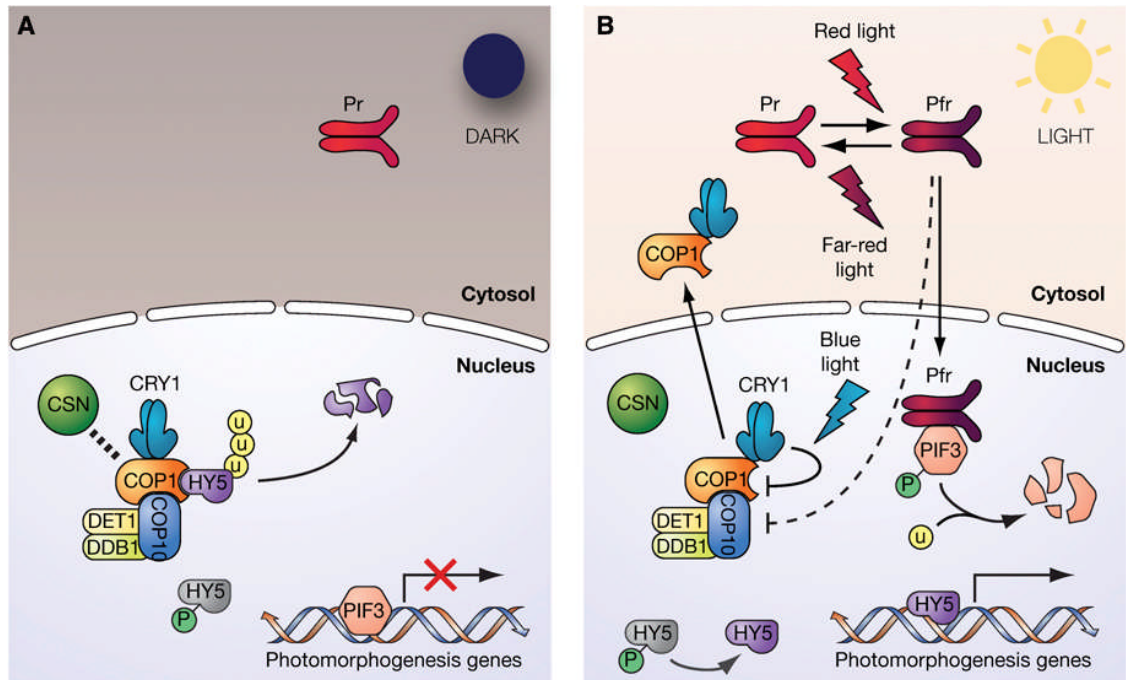
### ***Cytosolic signal transduction***

The HKRD of Pfr increases the phosphorylation level of, the cytosol-localised, phytochrome-kinase substrate 1 (PKS1) by around two-fold compared with Pr. The exact mechanism of HKRD and PKS1 interaction remains to be elucidated since HKRD is an atypical kinase (Fankhauser et al., 1999; Quail, 2002). PKS1 has been proposed as a negative regulator of phytochrome signalling, inhibiting nuclear translocation, since plants overexpressing PKS1 exhibit elongated hypocotyls (Fankhauser et al., 1999). Another protein, interacting at a different C-terminal domain, is nucleoside-diphosphate kinase 2 (NDPK2), it is reversibly phosphorylated by in R mediated by phytochrome (Tanaka et al., 1998). The phytochrome-NDPK2 interaction has a cytosolic and nuclear component, and there is preferential binding with the Pfr form, which increases NDPK2 activity by 70%. The phosphorylation of NDPK2 is thought to positively regulate the phytochrome signalling events, since *ndpk2* mutants have defects in cotyledon opening and apical hook straightening (Choi et al., 1999; Kreslavski et al., 2009).

### ***Transcriptional control***

The majority of phytochrome interacting proteins localise to the nucleus, one of these, phytochrome-interacting factor 3 (PIF3), is a member of the basic helix-loop-helix (bHLH) superfamily of transcriptional regulators and it physically interacts with Pfr via the C- and N-terminal domains (Ni et al., 1999) executing the primary mechanism of phytochrome signal transduction (fig. 1.1.1.2) (Franklin & Quail, 2009; Schäfer & Bowler, 2002; Quail, 2002). Pfr interacts with other members of the bHLH superfamily such as other PIFs (Huq & Quail, 2002), ARR4 (Sweere et al., 2001) or form dimers with PIF3 and HFR1 (Fairchild et al., 2000; Schäfer & Bowler, 2002). The bHLH superfamily proteins are transcription factors that recognise the E-box motif: CANNTG, the most common type of E-box in *A. thaliana* is the G-box: CACGTG (Martínez-

García et al., 2000). The G-box motif has been found in a number of promoter regions of light-regulated or light-responsive genes such as *chlorophyll a/b binding protein* (CAB) and *ribulose-1,5-bisphosphate-carboxylase/oxygenase small subunit* (RBCS) (Gilmartin et al., 1990; Terzaghi & Cashmore, 1995), genes which regulate or influence the rates of photosynthesis. The interaction of bHLH transcription factors with light-regulated genes, gives credence to the theory that phytochrome-bHLH binding is the first step in initiating de-etiolation (Franklin & Quail, 2009). PIFs and other members of the bHLH superfamily are negative regulators in the phytochrome signalling pathway. bHLH proteins are rapidly degraded in the light (Bauer et al., 2004; Monte et al., 2004; Monte et al., 2007), which reduces the support for any model with PIFs as positive regulators of phytochrome signalling. However *pif3* mutants exhibit delayed chloroplast development early in de-etiolation, after the initial exposure to light (Monte et al., 2004). PIF3 appears to have a role in mediation of the first stages of de-etiolation. In the dark, PIFs bind to G-boxes (Martínez-García et al., 2000) acting as repressors of genes involved in de-etiolation (fig. 1.1.1.2). In the light, Phy Pfr mediated degradation of PIFs via the ubiquitin-26S proteasome system (Park et al., 2004) would permit the rapid activation of de-etiolation (Duek & Fankhauser, 2005; Monte et al., 2007; Franklin & Quail, 2009). Similarly, a basic leucine zipper (bZIP) transcription factor, which binds G-boxes (Andronis et al., 2008; Chattopadhyay et al., 1998; Oyama et al., 1997) and is a positive regulator of photomorphogenesis (Chory, 1992), Long Hypocotyl 5 (HY5) is degraded in the dark by interaction with Constitutive Photomorphogenesis 1 (COP1), part of the COP9 signalosome (CSN) mediating ubiquitin-26S-directed proteolysis (Osterlund et al., 2000). In the light COP1 is translocated, following the interaction of cryptochromes and phytochromes (Yang et al., 2001), from the nucleus to the cytosol (von Arnim & Deng, 1994); HY5 accumulates so that it can act as a positive transcriptional regulator of photomorphogenic processes (fig. 1.1.1.2) (Waters & Langdale, 2009; Chory, 1992; Andronis et al., 2008).



**Figure 1.1.1.2 Simplified model of light signalling events during photomorphogenesis**

In the dark, the COP9 signalosome (CSN), a mediator of ubiquitin-26S proteolysis is active with the presence of COP1 in the nucleus. HY5, a bZIP transcription factor and positive regulator of photomorphogenesis associated genes via G-boxes, is targeted by CSN and COP1 for ubiquitin-26S directed proteolysis. PIF3, a bHLH transcription factor and negative regulator of photomorphogenesis associated genes repressing expression. In the light, phytochromes are converted to Pfr and interact with PIF3 promoting its degradation, freeing the G-boxes. Cryptochrome, CRY1, inactivates COP1 and promotes its translocation to the cytosol, thereby relieving the inactivation of HY5. The binding of HY5 to the G-boxes of photomorphogenesis associated genes brings about the process of de-etiolation (Waters & Langdale, 2009; Franklin & Quail, 2009; Duek & Fankhauser, 2005; Monte et al., 2007). Figure reproduced from figure 1 in (Waters & Langdale, 2009).

### ***Fast responses (ion channels and G-proteins)***

The role of phytochromes in modulating transcriptional events is without doubt. Whilst rapid phytochrome-mediated changes in light-regulated transcriptions have been recorded occurring within eight minutes (Salter et al., 2003), there are however light-dependent responses that are too rapid to be explained by changes at the transcriptional level. Examples of such responses include protoplast turgor (Shacklock et al., 1992), chloroplast light alignment (Serlin et al., 1996), changes in membrane potential (Ermolayeva et al., 1996), cytoskeleton reconfiguration (Takagi et al., 2003) and ethylene synthesis (Kreslavski et al., 1997; Kreslavski et al., 2009). Some fast responses: phototropism, chloroplast phototaxis, stomatal movements and changes in intracellular  $\text{Ca}^{2+}$ , are phototropin mediated (Harada et al., 2003). Ion channels are a universal mechanism of fast signalling and their states may be altered through substrate-receptor or ligand binding or be voltage-dependent (e.g. membrane depolarisation) (Medvedev, 2005). Light is able to trigger the opening of  $\text{Ca}^{2+}$ -permeable slow vacuolar channels via membrane depolarisation caused by  $\text{Cl}^-$  channel activation (Spalding, 2000). It is important to note that a fraction of the phytochrome pool is bound to the plasma membrane (Nagy & Schäfer, 2000), so this supports previous conclusions that exogenous phytochrome could rapidly depolarise artificial phospholipid bilayer membrane depending on its Pr/Pfr form (Roux & Yguerabide, 1973) and R-induced depolarisation of the plasma membrane in moss (Ermolayeva et al., 1996) therefore directly interacting with ion exchange proteins. However the existence of a GTP binding protein (G-protein) specific to phytochrome that would provide a basis of this mechanism has yet to be found (Maheshwari et al., 1999; Kreslavski et al., 2009). There is reason to suggest interaction with other secondary messengers: GTPases / guanine nucleotide-binding proteins (G-proteins) (Assmann, 2002; Nagy & Schäfer, 2000) and phosphatidylinositols, e.g. inositol-1,4,5-triphosphate ( $\text{IP}_3$ ) (Chen et al., 2008) and links to the  $\text{Ca}^{2+}$ -calmodulin (CaM) dependent protein kinases (Zielinski, 1998). The signalling cascades, which these secondary messengers trigger, mediate physiological and transcriptional responses to environmental, but specifically here, light stimuli (Kreslavski et al., 2009).

### ***Cryptochromes (blue and UV-A perception)***

The cryptochromes are another class of photoreceptor found in the animal and, ubiquitously, in the plant kingdoms. They perceive light at the blue (400 – 500nm) and UV-A portions of the spectrum (Cashmore et al., 1999). There are two main cryptochromes: CRY1 and CRY2 (Li & Yang, 2007). Their absorption spectra are

similar to flavin molecules, and they share considerable sequence homology in their N-terminal photolyase-related (PHR) domain with flavoprotein photolyases, which are responsible for mediating light-dependent repair of UV-B damaged DNA, e.g. pyrimidine dimers. This suggests an evolutionary development from photolyases to cryptochromes (Li & Yang, 2007; Cashmore et al., 1999). Cryptochromes are structurally differentiated from photolyases by a C-terminal domain, absent from photolyases. However it is the N-terminal domain of cryptochromes that confers the function of the molecule. The cryptochrome light signalling mechanism involves the homodimerisation of COP1 to the N-terminal domain (Li & Yang, 2007), mediating the translocation of COP1 from the nucleus to the cytosol and potentially facilitating the release of HY5 from COP1 (fig. 1.1.1.2) so that light-inducible genes are expressed via HY5 (Schäfer & Bowler, 2002; Waters & Langdale, 2009). Cryptochromes mediate anthocyanin production in response to blue light as well as contributing towards the development of de-etiolation (Franklin et al., 2005; Waters & Langdale, 2009).

### ***Phototropism and phototropins***

Phototropism, that is growth towards the direction of light, is in response, primarily, to blue light. Although not the first to describe the phenomenon, phototropism, like photomorphogenesis, was also investigated by Charles Darwin (Celaya & Liscum, 2005). However it was until the latter half of the 20<sup>th</sup> century that the biochemical (Short & Briggs, 1990) and genetic basis (Reymond et al., 1992) of phototropism was elucidated (Celaya & Liscum, 2005). Cryptochrome double mutant *cry1cry2* exhibits no loss in phototropic responsiveness. This indicates that phototropism is conferred by a separate set of blue light sensing proteins, termed phototropins (Laseve et al., 1999). The existence of two phototropins was discovered through the identification of a mutant for *PHOT1*, which whilst lacking phototropism in response to low fluence blue light, continued to respond to high fluence blue light (Liscum & Briggs, 1995). The locus of a second phototropin *PHOT2* was discovered and subsequent experiments with *phot1phot2* double mutants phototropism was severely impaired (Sakai et al., 2001; Franklin et al., 2005). Transgenic expression of NPH1/PHOT1 protein in insect cells revealed that the protein become phosphorylated in response to blue light; additionally recombinant NPH1 binds to flavin mononucleotide (FMN) (Christie et al., 1998; Celaya & Liscum, 2005). Phototropins are ~120kDa proteins, responsible for the ability of plant to detect the direction of light. They contain a PAS-like domains, with interactions with light, oxygen and voltage (LOV), interact with a flavin mononucleotide (FMN)

that acts as a chromophore providing the UV-A / blue light sensitivity of the phototropin (Christie et al., 1999; Celaya & Liscum, 2005).

### **Summary**

Plants have a variety of photoreceptors which allow them to perceive the quantity, quality, periodicity and direction of the light that they receive. Phytochromes, divided into five distinct proteins, perceive the R and FR wavelengths and are responsible for mediating photomorphogenesis (phyA) and responses to changes in the quantity and periodicity of light (phyB – E). The topic of responses to low light will be discussed in Section 1.2.1. Cryptochromes, another set of photoreceptors sensitive to the blue and UV-A portions of the spectrum, additionally mediate photomorphogenesis and entrainment of the circadian clock. Phototropins are also sensitive to the blue and UV-A wavelengths, however they are distinct from the cryptochromes and mediate phototropism. Signal transduction of light signals is greatly reliant on bHLH transcription factors, especially the PIFs, and their interaction with the phytochromes in providing transcriptional regulation of light-responsive genes. Faster responses are mediated through ion channels,  $\text{Ca}^{2+}$  and G-protein signalling in the cytosol and plasma membrane.

### **1.1.2 Low temperature responses**

As with light, temperature is an important environmental stimulus for plants to monitor. For poikilotherms, such as plants, the ambient temperature dictates the rate biochemical reactions, membrane fluidity and proper protein folding. Temperature at either extreme of the normal tolerance of plant can result in damage, so it is vital for its survival that a plant is sensitive to changes in temperature and respond rapidly. This study only considers gene regulation at warm and low temperature, so the effects and response to high temperature will not be discussed here. The adverse effects of low temperature on plants are above freezing, i.e. chilling, and freezing stress.

#### ***Effects of chilling***

Low temperatures above freezing are injurious and represent a stress for some plants, especially those from tropical climes. Innate and induced tolerances to low temperature vary greatly between plant species, which means providing a universal definition of chilling stress difficult. *Arabidopsis thaliana* are chilling tolerant, and do not sustain above freezing damage, but do undergo cold acclimation (Tokuhisa et al., 1997). Discussion of the injuries sustained by chilling sensitive species is useful to understand the processes of freezing tolerance. Chilling stress can be understood as injury or damage of one kind or another that plants sustain at temperatures from 10 or 12°C to freezing (Lyons, 1973; Levitt, 1980a). Injury due to chilling may be divided into direct, indirect and secondary damage. Direct injuries result from sudden, brief cold shocks close to freezing, which are associated with membrane permeability from pseudoplasmosis and electrolyte leakage (Patterson et al., 1976; Levitt, 1980a). Indirect damage arises from gradual, sustained chilling causing metabolic disturbances reducing rates of aerobic respiration and photosynthesis (Labate & Leegood, 1988), toxin accumulation e.g. reactive oxygen species (ROS) (Foyer et al., 1997), metabolic deficits, e.g. of ATP, which results in decreased ion uptake and protein synthesis. Secondary effects, also resulting from gradual and sustained chilling, cause crises such as water stress, even though transpiration is much reduced, due to poor water uptake from the roots (St. John & Christiansen, 1976; Levitt, 1980a).

#### ***Effects of freezing injury***

Injuries from freezing temperatures arise from two effects: dehydration and membrane damage. From 0°C ice begins to form in the intercellular spaces first, since the cytoplasm has a much high solute potential hence a high freezing point. This

extracellular freezing is facilitated by the presence of heterogeneous nucleation agents such as dust and bacterial proteins (Xin & Browse, 2000). Homogeneous ice-nucleation occurs at  $-32^{\circ}\text{C}$ , above which water remains in a supercooled state (Thomashow, 1998). Water in the form of ice has a much lower water potential than liquid water and the differential increases as temperature decreases. The extracellular ice therefore has a dehydrating effect on plants, such that water moves out of the cells via osmosis (Thomashow, 1998; Xin & Browse, 2000); at  $-10^{\circ}\text{C}$  more 90% of intracellular liquid will move out into the intercellular space, leaving a balanced osmolarity (Osm) of 5 Osm. Freeze-induced dehydration is likely to cause damage via the denaturation of proteins and the precipitation of various molecules (Thomashow, 1998) provoked by the change in osmolarity. This would severely impair cells to carry out the biochemistry required to sustain life. Freezing temperatures cause membrane lesions – ultrastructural changes to the plasma membrane, such as the appearance of hexagonal II phase in the plasma membrane and subtending lamellae (Uemura et al., 1995). These ultrastructural changes are associated with increased electrolyte leakage and cell death in cereal protoplasts.

### **Cold acclimation**

Plants of temperate climates are exposed to seasonal variation in temperature which may range between  $+40$  to  $-30^{\circ}\text{C}$ . Wheat and rye are able to survive temperatures as low as  $-20$  and  $-30^{\circ}\text{C}$  respectively. However this freezing tolerance is not seen in wheat and rye grown at warm temperatures. It is only after a period of cold acclimation – growth at low, but non-freezing temperatures, that this freezing tolerance seen (Thomashow, 1994; Thomashow, 1998). Freezing tolerance is not acquired until plants have undergone a period of low temperature above freezing (Gilmour et al., 1988; Levitt, 1980b). This period of chilling that improves freezing tolerance, known as cold acclimation, is associated with broad alterations to membrane structure, gene expression and associated downstream changes in metabolism and cellular function mediating freezing tolerance. Freezing injury occurred at different temperatures depending on whether protoplasts received cold acclimation first (Webb et al., 1994).

### **Membrane changes**

Low temperatures provoke a transition in membranes from a liquid crystalline state to a gel-like phase and with it a reduction in membrane fluidity. Maintenance of membrane fluidity is crucial for the normal function of essential membrane proteins, which otherwise become deactivated (Upchurch, 2008). In cold acclimated *A. thaliana*,

freezing conditions, which caused membrane damage to non-acclimated protoplasts, resulted in none of the observed membrane ultrastructural changes, which cause membrane destabilisation. A week of cold acclimation at 2°C resulted in a significant modification to the lipid composition of the plasma membrane to the effect of increasing the proportion of unsaturated fatty acids and sterols (Uemura et al., 1995). Such changes will maintain membrane fluidity and the lower temperatures, reducing the incidence of membrane destabilisation (Watanabe et al., 1990; Falcone et al., 2004). During abiotic stress, especially osmotic and cold stress, there is an accumulation of compatible solutes, which in plants include carbohydrates, such as Glc, Fru and Suc; amino acids and derivatives, such as proline (Pro), glycinebetaine and polyamines (Yancey et al., 1982; Alberdi & Corcuera, 1991; Hare & Cress, 1997). The cryoprotectant properties of compatible solutes extended beyond their effects on the colligative properties of a solution; compatible solutes interact with membranes improving their stability at low and freezing temperatures. Carbohydrates interact with the head groups of phospholipids, whereas amino acids and their derivatives take part in hydrophobic interactions within the bilayer (Anchordoguy et al., 1987).

### **Changes in Cold Regulated (COR) gene expression**

The identification of seven mutants *sensitive to freezing 1 – 7 (sfr1-7)* provided a clearer genetic basis for the acquisition of freezing tolerance. Mutants *sfr3*, *4*, *6* and *7* were deficient or had entirely absent accumulation of anthocyanin in response to during cold acclimation; *sfr4* did not accumulate carbohydrates; *sfr4* and *sfr7* affected fatty acid composition after cold acclimation; *sfr1*, *sfr2* and *sfr5* had no altered biochemistry compared with wild-type despite being freezing sensitive (McKown et al., 1996). The *sfr6* mutants were found to be deficient in their expression of cold-inducible genes *KIN1*, *COR15a*, *LTI78*. These genes are regulated via the CRT/DRE motif; the *sfr6* exhibited normal cold-induced expression of *CBFs 1-3*, which lack the CRT/DRE motif (Knight et al., 1999). Expression of *COR15a*, whose protein is targeted to the chloroplast outer membrane, reduces the propensity for the formation of hexagonal II phase membrane lesions associated with freezing damage. After cold acclimation in winter cereals the formation of the hexagonal II phase lesion is prevented, however overexpression of *COR15a* without cold acclimation only delays lesion formation to a lower temperature. Clearly other factors, such as lipid composition and compatible solutes accumulation, are necessary in cold acclimation (Steponkus et al., 1998). Chromatin remodelling has been implicated in the genome-wide transcriptional changes in response to low temperature. The expression and activity of a number of histone

modification enzymes, chromatin binding proteins and other chromatin remodelling factors is affected by low temperature and other abiotic stresses (Kim et al., 2010). Given that cold acclimation is required for the development of freezing tolerance, the sensing of low temperature must be associated with signalling processes upstream of its regulation. (Guy et al., 1985) were the first to report altered gene expression during cold acclimation and since the early 1990s a number of cold-regulated genes have been described and designated *cold regulated (COR)*, *low-temperature-induced (LTI)*, *kykna-indusoitu (KIN)* (Finnish meaning: cold-induced) (Thomashow, 1994), *responsive to dessication (RD)* or *early dehydration-inducible (ERD)* (Thomashow, 1998). An early described *COR* gene was *COR47* that coded for a protein which remained stable following boiling in an aqueous solution, it was suggested that such a protein was a cryoprotectant (Lin et al., 1990). Another, *COR15a*, was found to code for a protein with cryoprotectant qualities and stabilised the cold-labile enzyme lactate dehydrogenase against inactivation with greater efficacy than either Suc or bovine serum albumin (BSA) (Lin & Thomashow, 1992). Genome-wide gene expression assay with ~24,000 genes represented, detected 939 cold-regulated genes with 655 (69.8%) up-regulated in response to cold (0°C) and 248 (30.2%) down-regulated by low temperature (Lee et al., 2005). The majority of the cold-regulated genes identified by (Lee et al., 2005) are late-responsive, that is responding between at the 24 hour interval; 66.4% of the up-regulated genes and 91.5% of the down-regulated genes were late-responders. There is considerable redundancy in the cold-regulated genes (Xin & Browse, 2000): mutants of two cold-regulated genes involved in anthocyanin biosynthesis: *phenylalanine ammonia lyase* and *chalcone synthase* do not have impaired freezing tolerance (Leyva et al., 1995). Certain cold-regulated genes however do seem to play a prominent role in mediating cold acclimation (Xin & Browse, 2000): constitutive expression of *COR15a* confers enhanced *in vivo* freezing tolerance to non-acclimated plants and *in vitro* survival of non-acclimated protoplast compared with wild-type (Artus et al., 1996).

## **Cold Sensing**

### **Membrane mediated**

The observed changes in membrane lipid composition in response to low temperature may also be a site of temperature perception. Chemical modulation of membrane fluidity using membrane 'rigidifier' Dimethyl sulphoxide (DMSO) and membrane 'fluidizer' Benzyl alcohol (BA) was found to induce cold acclimation without cold treatment or reduce the development of cold acclimation and therefore reduce freezing tolerance respectively. Similarly, artificial membrane fluidisation prevents the cold-induction of cold up-regulated transcripts, whilst artificial membrane rigidification was able to induce cold up-regulated transcripts and  $\text{Ca}^{2+}$  influx at 25°C with associated increases in freezing tolerance (Örvar et al., 2000). The state of actin filaments, also reported by Örvar et al., (2000), influences the development of freezing tolerance: artificial stabilisation of actin filaments at 4°C prevents  $\text{Ca}^{2+}$  influx and the induction of cold-regulated transcripts; artificial destabilisation of actin at 25°C had the opposite effect.

### **Calcium signalling**

The first definitive evidence for the role of calcium signalling in low temperature responses came with the innovation of plants expressing aequorin, a calcium-sensitive protein originating from the cnidarian, *Aequoria victoria*, allowing the measurement of changes in  $\text{Ca}^{2+}$  flux. The transition of seedlings from 20°C to 5 or 0°C was associated with a strong increase in the intracellular calcium concentration, which could be increased further when 10mM  $\text{Ca}^{2+}$  was applied to the chilling medium (Knight et al., 1991). Treatments with an antagonist of calcium-binding protein, calmodulin, and of calcium-dependent protein kinases or the  $\text{Ca}^{2+}$  channel blocked lanthanum ( $\text{La}^{3+}$ ) resulted in a decrease in cold-induced protein phosphorylation and an abolition of freezing tolerance (Monroy et al., 1993). The majority of extracellular calcium is found in the apoplast attached the cell wall (Cleland et al., 1990). More sensitive aequorin measurements found that  $\text{La}^{3+}$  and EGTA were only able to partially inhibit  $\text{Ca}^{2+}$  influx and cold-induced gene *KINI* suggesting an intracellular store of  $\text{Ca}^{2+}$ . Aequorin targeted to the vacuolar membrane confirmed that the vacuole was indeed the source of intracellular  $\text{Ca}^{2+}$  release. Treatments with inhibitors of inositol trisphosphate ( $\text{IP}_3$ ), indicated a role for vacuolar membrane localised pool of this secondary messenger in mediating  $\text{Ca}^{2+}$  release into the cytosol (Knight et al., 1996). Calcium does not bind DNA directly but signals are transduced via the binding of proteins with affinity for

calcium such as calmodulin (CaM) and analogous molecules (Bouché et al., 2005) as well as interacting with calcium-dependent kinases (Harper et al., 2004; Kaplan et al., 2006).

### **The COR/CBF/ICE Pathway**

Transcript levels of *COR* genes increase rapidly and strongly within four hours of moving plants to 5°C and peak around 12 hours and remain highly expressed until restored to 22°C (Hajela et al., 1990). The first cis-acting regulatory element responsive to drought, low-temperature or high salinity was called the drought responsive element (DRE) with the nine base-pair (bp) motif: TACCGACAT. This motif was bound by, then uncharacterised nuclear proteins, which induced expression in genes *RD29a* (*COR78*) and *RD29b* (*LTI65*) (Yamaguchi-Shinozaki & Shinozaki, 1994). One such nuclear protein and its associated gene were characterised and termed C-repeat/DRE binding factor 1 (CBF1), binding a core motif of CCGAC. CBF proteins possess an APETELA (AP2) domain, which has a DNA-binding region that may bind the CRT/DRE (Stockinger et al., 1997). Overexpression of *CBF3* (*DREB1A*), whose expression is cold-inducible, results in strong induction of its target genes under non-stress condition, dwarfed phenotypes and tolerance to freezing and drought (Liu et al., 1998). A 125bp region of the *CBF2* promoter was found to be sufficient to confer cold-responsive gene expression; mutation analysis revealed motifs required for cold responsiveness, designated inducer of CBF expression (ICE) boxes (Zarka et al., 2003). The existence of ICE boxes recognised by ICE transcription factors was hypothesised by (Gilmour et al., 1998) since *CBF* transcripts accumulated within 15 minutes, suggesting a pre-existing transcription factor mediating *CBF* cold-induction. An *ICE1* locus was identified and with it, from an ethylmethane sulfonate (EMS) mutagenesis screen, an *ice1* mutant which exhibits almost no *CBF3::LUC* or native *CBF* expression. Expression of downstream targets of *CBF* genes, *COR15a* and *COR47a*, were also much reduced in *ice1* mutants. The warm-ground phenotype of *ice1* was little changed from wild-type however, after cold acclimation the freezing tolerance of *ice1* was impaired compared to wild-type. The *ICE1* gene was found to code for a MYC-like basic-helix-loop-helix (bHLH) transcription factor. Expression analysis using *ICE1::GUS* revealed a constitutive expression in the roots, leaves, stems and inflorescences but with stronger localisation to leaves and stems. *ICE1* expression is mildly induced by cold, salt and ABA, but insensitive to dehydration. ICE1-GFP revealed a subcellular to the nucleus independent of temperature. *ICE1* is a transcriptional activator of *CBF* expression (Chinnusamy et al., 2003). These findings

suggest that post-translation modification of ICE1 is required in order for activation of downstream genes (Chinnusamy et al., 2007). ICE1 directly interacts with MYC sites, which are found in the promoter of *CBF3* (Chinnusamy et al., 2003). MYC-like bHLH transcription factors form a complex with MYB transcription factors in order to activate transcription (Spelt et al., 2000) and numerous MYB and MYC binding sites have been identified in the promoters of *CBF1-3* (Shinwari et al., 1998). Analysis of the cold-responsive transcriptome, carried out by (Lee et al., 2005), revealed that in the *ice1* mutant there was altered expression of: transcription factor genes notably of the *bZIP* and *WKRY* classes; genes involved in Ca<sup>2+</sup> signalling, lipid signalling and receptor-like protein kinases.

### **Negative regulation of the COR/CBF/ICE pathway**

The CBF transcription factors negatively feedback onto each other; the *cbf2* null mutant exhibits greater freezing tolerance arising from greater levels of *CBF1* and *CBF3* compared with wild-type; complementation of the mutant with *CBF2* behind its native promoter restored wild-type expression of *CBF1* and *CBF3*, confirming *CBF2* as a negatively regulator of *CBFs 1* and *3*. Transcripts of *CBF1* and *CBF3* begin to accumulate within 15 minutes and peaking after 90 minutes then rapidly declining, whereas *CBF2* accumulates more slowly and peaks after two-and-a-half hours followed by a gradual decline (Novillo et al., 2004). Using a similar rationale the lack of *CBF3* expression in the *ice1* mutant and increased levels of *CBF2* (Chinnusamy et al., 2003) could make *CBF3* a repressor of *CBF2* (Chinnusamy et al., 2007). As noted above ICE-boxes / MYC sites are found along site MYB transcription factor binding sites in the promoters of *CBF* genes; such a transcription factor, *MYB15*, is up-regulated during cold stress and binds to MYB sites in *CBF* promoters. *MYB15* interacts with ICE1 as revealed by yeast-2-hybrid and co-localises to the nucleus. Overexpression of *MYB15* resulted in a reduction in *CBF* gene expression under cold stress and concomitant impairment of freezing tolerance. A *myb15* mutant had increased *CBF* gene expression and elevated expression was seen in CBF downstream *COR* gene targets; *myb15* had increase freezing tolerance compared to wild-type (Agarwal et al., 2006). These results indicate *MYB15* as an upstream negative regulator of *CBFs* (Chinnusamy et al., 2007). A cold-induced C2H2 zinc finger transcription factor gene, *ZAT12* has been implicated as a negative regulator of *CBFs* (Chinnusamy et al., 2007); following the peak in *CBF* expression after two hours there is induction of *ZAT12* expression – pursuant is a swift decline in *CBF* transcript. Overexpression of *ZAT12* reduced the levels to which *CBF1*,

2 and 3 accumulated in result to low temperature; downstream target of the *CBFs* were marginally decreased from wild-type levels (Vogel et al., 2004).

### **Abscisic acid signalling**

Experiments with *ABA insensitive (abi)* and *ABA deficient (aba)* revealed a role for ABA signalling in regulation of *COR* genes. The *abi* mutants had no discernable impairment in their cold acclimation and subsequent freezing tolerance, whereas the *aba* mutants were severely impaired in terms of their freezing tolerance compared to wild-type. Electrolyte leakage in *aba* mutants was much greater than in wild-type (Gilmour & Thomashow, 1991). Earlier studies revealed that ABA treatment at warm temperatures was able to provoke cold acclimation and the development of freezing tolerance (Lång et al., 1989) and ABA accumulates transiently in response to low temperature and strongly in response to drought (Chen et al., 1983; Lång & Palva, 1992; Lång et al., 1994). Expression of *CRT/DRE::LUC* is also induced by ABA treatments, responding within one hour and peaking after three. Expression of *CBF* genes 1 – 3 (*DREB1a, b, c*) was found to be inducible by ABA application; levels of transcript peaked after one hour and subsequently declined. *CBF* promoter  $\beta$ -glucuronidase (*GUS*) fusions had the same responsiveness to ABA as native *CBF* genes, indicating that ABA exerted its influence via transcriptional rather than post-transcriptional control (Knight et al., 2004). ABA signalling is mediated via the ABA response elements (ABRE) and the ABRE binding factors (ABF) (Choi et al., 2000). Whilst the ABRE is found in the promoters of some *COR* genes, others lack these elements and ABA signalling has been shown also act via the CRT/DRE (Knight et al., 2004). The role of ABA signalling in low temperature sensing has been questioned since ABA levels increase only transiently in response to low temperature (Chen et al., 1983) that endogenous ABA does not reach sufficient levels to implicate ABA in cold sensing (Shinozaki & Yamaguchi-Shinozaki, 2000). Knight et al., (2004) propose that ABA might potentiate the cold-induced CBF signalling even without ABA and cold signalling occurring simultaneously. Knight et al., (2004) offer an alternative hypothesis whereby there is ‘cross-talk’ between drought responsive ABA signalling that recruits the CBF transcription factors of the cold response pathway.

### 1.1.3 Sugar sensing and signalling

Just as light and temperature influence metabolism at a biochemical level, they also provide important environmental cues by which plants are able to direct their growth and development appropriately to their environment. So too then, are sugars more than just metabolites but are also indicators of energy status detected in order to regulate plant growth and development. Consonant with sugars having a role as regulators in metabolism, QTL analysis (Meyer et al., 2007) has revealed that there is an inverse correlation between growth rate and metabolite levels. This suggests that mere availability of metabolites does not determine growth rate, but rather a more subtle combination of developmental and physiological cues, with resource availability, modulates growth (Hanson & Smeekens, 2009).

#### **Glucose sensing**

Glucose (Glc) can be considered a universal carbon-energy molecule given its common role across diversity of life (Ramon et al., 2008). Glc delivers energy via glycolysis, but also acts as a 'carbon-skeleton' for the biosynthesis of larger molecules such as cellulose for cell walls (Moore et al., 2003) or functional sugars via the pentose-phosphate-pathway (Claeyssen & Rivoal, 2007). The first step in glycolysis is the phosphorylation of Glc to glucose-6-phosphate (G6P). Hexokinase (HXK) is the enzyme responsible for this and was first described in *Saccharomyces cerevisiae* (Meyerhof, 1927). Otto Meyerhof together with Gustav Embden and Jakub Karol Parnas fully described the eponymous Embden-Meyerhof-Parnas pathway more commonly known as glycolysis. HXK catalyses the phosphorylation, with ATP, of Glc to G6P, however as the name suggests it also has affinity for fructose (Fru), mannose (Man), and galactose (Gal) and is distinct from the kinases with single-hexose affinities (Claeyssen & Rivoal, 2007).

#### **Hexokinase**

The broad affinity of HXK renders the enzyme the primary entry to glycolysis and biosynthetic pathways for sucrose (Suc) and starch breakdown products (Claeyssen & Rivoal, 2007). It is this central metabolic position which HXK occupies that also makes it an ideal sensor of cellular energy status. The first evidence for sugars being involved in signalling pathways comes from findings that sugars were capable of repressing photosynthetic gene expression in maize mesophyll protoplasts in a concentration dependent manner (Sheen, 1990). Later work on *Chenopodium* corroborated this finding and additionally found that when exogenous carbohydrates had been exhausted,

the repression of photosynthetic gene expression began to be relieved and prior to any significant change in carbohydrate content. This suggested that sugar-induced repression was linked with either the transport or metabolism of sugar, rather than sugar *per se* (Krapp et al., 1993). The implication of hexokinase came when research discovered that genes induced during starvation conditions and repressed by Suc or hexose treatment, but also by 2-deoxyglucose (2-DG) and Man, which can be phosphorylated but do not contribute towards glycolysis (Graham et al., 1994). This suggests that sugar-regulated genes are HXK dependent (Graham et al., 1994); further supported by the fact that 3-O-methylglucose (3-OMG) and G6P, which are not substrates of HXK do not elicit repression (Jang & Sheen, 1994). In *A. thaliana* there are two HXK genes: *HXK1* and *HXK2* which are able to complement yeast *hvk1* and *hvk2* mutants respectively (Jang et al., 1997). Whilst sharing 82% nucleotide and 85% amino acid homology (Jang et al., 1997), *HXK1* and *HXK2* have distinct functions attested to by *glucose insensitive (gin)* mutants. *gin2-1* and *gin2-2* are null and missense mutants respectively, with *gin2-1* producing no *HXK1* protein at detectable levels, but with normal *HXK2* transcript levels. *gin2-1* mutants are not retarded in their development, but their leaves appear much smaller and of a darker green than wild-type, suggesting a role for *HXK1* in regulating growth. The exhibited mutant phenotype could be complemented with a *35S::HXK1* transgene, but not by *35S::HXK2*, demonstrating that *HXK1* is the Glc sensor in plants (Moore et al., 2003). In addition to *HXK1* and *HXK2* there are a further four *HXK* genes, with disparate localisations within the plant and expression profiles (da-Silva et al., 2001; Claeysen & Rivoal, 2007). *HXK* isoforms have been found to have a cytosol, mitochondrial and plastidic localisations: *HXK2* and At1g50460 were found attached to the outer mitochondrial membrane (Giegé et al., 2003). Studies have shown that *HXK1* has a nuclear (Yanagisawa et al., 2003) localisation and attachment to the chloroplast outer membrane (Wiese et al., 1999). Attachment to chloroplasts and mitochondria would facilitate the rapid acquisition of metabolites from starch breakdown (Wiese et al., 1999) and the transfer of the products of glycolysis to mitochondria for oxidative-phosphorylation (Yamamoto et al., 2000)

### **HXK Signal transduction**

The nuclear localisation of *HXK1* suggests direct role for this enzyme in the transduction of resource availability signals (Claeysen & Rivoal, 2007). This hypothesis is corroborated by the fact that Glc, via *HXK1*, promotes the proteasome-dependent degradation of transcription factor *ethylene insensitive 3* (*EIN3*)

(Yanagisawa et al., 2003). This finding was followed up with research demonstrating an interaction between HXK1, ATPase B1 (VHA-B1) and the 19S regulatory particle of proteasome subunit (RPT5B). This complex directly binds to the promoters of Glc-regulated genes in a Glc-dependent manner (Cho et al., 2006). Mutants of *vha-b1* and *rpt5b* phenocopy the HXK1 null, *gin2-1* (Cho et al., 2006) pointing to the requirement of the complex for HXK1-dependent Glc signalling (Hanson & Smeekens, 2009).

### ***HXK-independent pathways***

Other Glc-responsive genes such *ADP-glucose pyrophosphorylase (AGPase)*, *cell-wall invertase (CIN)*, *chalcone synthase (CHS)*, *phenylalanine ammonia-lyase (PAL)*, which are positively regulated, and *asparagine synthetase (ASN)*, which is negatively regulated, all maintained their wild-type responsiveness in plants over-expressing *HXK1* or expressing anti-sense *HXK1* transcripts. This finding demonstrated that HXK-independently Glc-regulated genes exist (Xiao et al., 2000). The HXK-dependent and – independent regulatory pathways sometimes cooperate in the regulation of the same gene. Expression of a monosaccharide transporter is repressed by Glc; regulation at the transcriptional level is HXK mediated, where at the post-translational level it is HXK-independent (Conde et al., 2006; Ramon et al., 2008). Plants, as in yeast and animals, use cell surface receptors to mediate extracellular signalling one these is the G-protein coupled receptors (GPCRs) (Chen et al., 2003; Rolland et al., 2006). GPCRs upon activation interact with G-proteins causing the  $\alpha$ -subunit (GPA1) to exchange guanosine-diphosphate (GDP) for guanosine-triphosphate (GTP). In addition to interacting with G-protein coupled receptor 1 (GPCR1), GPA1 also interacts with a seven-transmembrane domain protein, regulator of G-protein signalling 1 (RGS1). *rgs1* mutants exhibit increased GPA1 activity as well as hypocotyl elongation in darkness (Chen et al., 2003). The *rgs1* mutants were insensitive to 6% Glc, which for wild-type causes severe stunting of growth. These mutants exhibited wild-type response for sugar-analogues confirming the HXK-independence of this pathway (Chen & Jones, 2004; Chen et al., 2003; Rolland et al., 2006).

### **Kinase-mediated signal transduction**

In yeast glucose-mediated repression is relieved by the activity of a protein Ser/Thr kinase, sucrose-non-fermenting 1 (SNF1) (Smeekens, 2000). An equivalent kinase, SNF-related kinase 1 (SnRK1) has been found in plants (Halford & Grahame Hardie, 1998). In yeast SNF1 acts a transcriptional regulator of the genes involved in carbohydrate metabolism and expression of rye *SnRK1* was found to restore SNF1

function in *snf1* yeast mutants. Additionally expression of anti-sense *SnRK1* in potato was found to severely reduce expression of *sucrose synthetase* (Halford & Grahame Hardie, 1998). Further researched demonstrated that SnRK1 is involved in regulating sucrose biosynthesis, nitrogen assimilation and secondary metabolic pathways (Sugden et al., 1999). There is some dispute as to the regulation of SnRK1; Baena-González et al., (2007) report that a SnRK1 homologue KIN10, when activated, promotes genes of catabolic pathways ways such as the degradation of starch, sucrose and cell walls and represses genes involved in anabolism / biosynthesis. However (Halford & Hey, 2009) cast doubt on this, citing a 1998 article by Purcell et al. that describes results of the expression of anti-sense *SnRK1* in potato, to effect of decreased expression of *sucrose synthetase* in tubers and loss of sucrose-inducibility *sucrose synthetase* in leaves. Further conflicting evidence comes from tubers of potatoes overexpressing *SnRK1*, which had higher levels of starch and lower Glc, likely resulting from an increase in the expression of *sucrose synthase* and *AGPase* (McKibbin et al., 2006). Halford & Hey, (2009) point out that these findings are not compatible with the model put forward by Baena-González et al., (2007) of SnRK1 being activated under sugar-starvation conditions and repressed in times of plenty. Application of a comparatively low concentration (25mM) of the non-reducing disaccharide, trehalose (Tre) results in strong inhibition of seedling root growth with accumulation of starch in the shoots. The growth inhibition is relieved by the application of Glc or Suc alongside Tre. The accumulation of starch was associated with elevated AGPase activity, an enzyme which catalyses the first step in starch synthesis. Expression of *APL3*, an AGPase gene, is mildly induced by 50mM Suc, but this is further increased by the application of 5mM Tre (Wingler et al., 2000). Mutants of *trehalose phosphate synthase (tps1)*, which codes for an enzyme catalysing the synthesis of trehalose 6-phosphate (T6P) – the immediate precursor to Tre, are embryo lethal: arrested at the torpedo stage of development. This is the phase of embryo development associated with increases in Suc and the formation of storage reserves (Eastmond et al., 2002). Expression of *Escherichia coli TPS (otsA)*, in *tps1* mutants was able to rescue their embryo lethality. Seedlings expressing *otsA* accumulate anthocyanins and the rosettes of developing plantlets are smaller and darker green than wild-type; seed set is poor. The expression of *E.coli trehalose phosphate phosphatase (TPP) (otsB)*, which converts T6P to Tre, and *trehalose phosphate hydrolase (TPH) (treC)* which converts T6P to Glc and G6P, in wild-type background, both produce the same phenotype: delayed development and flowering, but as mature plants with larger, lighter green leaves than wild-type; seed set is plentiful. The growth

of *otsB* and *treC* seedlings was severely inhibited by addition of Glc or Suc to the medium, whereas growth of *otsA* is accelerated over wild-type with sugar supplementation. This indicated that T6P is crucial for the carbohydrate utilisation (Schluepmann et al., 2003). Levels of T6P increase with Suc and Tre feeding, the pool of hexoses also increases with Suc treatment, the changes in T6P levels in response to Suc, place T6P in a role as a signalling molecule for energy status. The growth arrest seen with the application of Tre is caused by the accumulation of T6P (the conversion of T6P to Tre is irreversible), since *treC* expression overcomes the effects of T6P supplementation by converting it back to G6P and Glc (Schluepmann et al., 2004). Furthermore, any growth inhibition caused by T6P accumulation is relieved by the application of metabolisable sugar. The role of T6P as a signalling molecule is indicated by the correlation of levels of T6P with the expression of *KIN11*, a gene for of the catalytic subunits of SnRK1 (Fragoso et al., 2009), such that expression of *KIN11* is elevated with high levels of T6P and reduced when T6P levels are low (Schluepmann et al., 2004). T6P at micromolar concentrations, which reflect *in vivo* levels, was found to be an inhibitor of SnRK1 (KIN10/11) protein activity, requiring a thousandth the concentration of G6P to achieve the same level of inhibition. The inhibition of SnRK1 by T6P requires an intermediate not found in mature plant leaf extract. The lack of T6P inhibition of SnRK1 in mature tissue may mean that the other role for T6P in regulating starch synthesis is predominant in these tissues. Taken together, these findings place T6P in a role of signalling energy status, inactivating SnRK1, which mediates the sugar-starvation responsive inhibition of biosynthesis genes, thereby promoting growth (Zhang et al., 2009).

### ***Sucrose sensing and signalling***

Since Suc is readily degraded to hexoses by invertase, the search for a Suc sensing mechanism has been difficult to uncouple from that of the hexoses. However use of Suc analogues, palatinose and turanose, demonstrated repression of  $\alpha$ -amylase via another method than seen with Glc and Suc treatments, which cause destabilisation of  $\alpha$ -amylase transcript (Loreti et al., 2000). Many specifically Suc regulated genes have been identified (Gonzali et al., 2006), along with evidence of Suc regulated gene expression and signalling independent of hexoses (Ramon et al., 2008). Investigation of a light regulated gene *ATHB2*, which encodes an S-class bZIP transcription factor bZIP11, found that Suc was able to repress the activity of an *ATHB2*-promoter-*GUS* (*ATHB2::GUS*) activity, with hexoses and other disaccharides having no effect. It was found that levels of native *ATHB2* and *ATHB2::GUS* transcripts were not repressed, but

in fact upregulated, meaning that Suc has its effect on the post-transcriptional level (Rook et al., 1998). Further investigation revealed that regulation was at the translational level, focussed on a small upstream open reading frame (uORF) in the 5' UTR, which is conserved amongst the S-class bZIP transcription factors (Rook et al., 1998; Wiese et al., 2004; Ramon et al., 2008).

### ***Sugar-regulated transcription***

The responsiveness to sugars is not regulated exclusively at the transcriptional level, but there are many genes that are regulated via sugar responsive elements found in their promoter regions. Sucrose-responsive elements (SURE) were identified in the promoter of potato tuber storage protein gene, *patatin*. SUREs have similarity to sporamin promoter motif-8 (SP8) and SUREs are bound by sucrose response factors and both SUREs and SP8 motifs are bound by SP8BF rather than any direct interaction with sucrose *per se* (Grierson et al., 1994). Other sugar responsive cis-acting elements include A- and B-boxes, and the TGGACGG element (Rolland et al., 2006). A WRKY-like transcription factor, SPF1 binds to SP8 motifs acting as sucrose-repressed, negative regulator of gene expression (Rolland et al., 2006). Others of this type also bind W-boxes and SUREs (Rolland et al., 2002; Rolland et al., 2006). Sugars modulate the expression of plant hormones such as abscisic acid (ABA) (Arroyo et al., 2003) and ethylene biosynthesis (Rolland et al., 2006). Transcripts of *abscisic acid insensitive: ABI4* and *ABI5*, which encode transcription factors of the APETALA 2 (AP2) domain and bZIP family respectively, accumulate at high (7% w/v) levels of Glc supplementation, but not at lower levels (2% w/v) (Arroyo et al., 2003; León & Sheen, 2003). The induction of *ABI4/5* by Glc is in an ABA-dependent fashion (León & Sheen, 2003). *abi4* and *abi5* mutants are insensitive to Glc exhibiting no stunting of growth or development at 7% w/v Glc (Arroyo et al., 2003); additionally, overexpression of *ABI5* results in sugar hypersensitivity (León & Sheen, 2003).

ABA-response element (ABRE) binding factor 2 (*ABF2*), a bZIP class transcription factor (recognising the cis-element CACGTG) is a repressor of genes upregulated by ABA signalling. Overexpression of *ABF2* results in a reduction in ABA and Glc sensitivity. The *abf2* null mutant has a defective Glc response and lacking inhibition, grows faster (Kim et al., 2004). The rice *α-amylase* gene is repressed by sugars and linker-scan analysis, whereby portions of the promoter were replaced by an EcoRI restriction site, isolated a sugar response sequence (SRS) in the promoter containing a

GC-box, a G-box and a TATCCA element (Lu et al., 1998). Lu et al., (1998) found that the SRS was able to render a sugar-insensitive actin gene responsive to sugar and the presence of a GC-box with either a G-box or a TATCCA element would mediate a sugar response. The TATCCA element is required for high level responses to sugar and GA, mutations of the element result in declines of up to 20% in responsiveness (Lu et al., 1998). The genes *OsMYBS1*, 2, and 3 encode MYB transcription factors that bind the TATCCA element, recognising the core TATCC. OsMYB1 and 2 are transcriptional activators, with OsMYB1 exhibiting a strong effect and OsMYB2 only weakly; OsMYBS3 appears to have no such function. OsMYBS1 and 2 both induce expression of a reporter gene with a promoter containing SRS with or without sugar, suggesting transcriptional control. OsMYBS2 is expressed highly and binds to TATCCA when sugar is present, but only induces low level expression of  *$\alpha$ -amylase*. Under sugar starvation conditions, *OsMYB1* is expressed highly with a concomitant suppression of *OsMYBS2*. OsMYBS1 binds TATCCA inducing high level  *$\alpha$ -amylase* expression. OsMYBS3 is most abundant in senescing leaves, so OsMYBS3 may block  *$\alpha$ -amylase* expression. The TATCCA is also a necessary cis-element in  *$\alpha$ -amylase* genes regulated by GA, along with a GARE sequence upstream of TATCCA, which is bound by HvMYBGa. GA signalling is inhibited by ABA and these two hormones have opposite effect on OsMYB1 and 2: GA suppresses OsMYB1 accumulation but has no effect on OsMYB2; ABA suppresses OsMYB2 but has no effect on OsMYB1. In the presence of GA  *$\alpha$ -amylase* promoter activity was with either OsMYB1 or OsMYB3 but was maintained by OsMYB2 and even further by HvMYBGa (Lu et al., 2002).

### **Summary**

Plants are able to detect sugars either as hexoses or sucrose, such ability provides important information for the monitoring of the resource availability so that growth and development may proceed appropriately. Plants can detect hexoses in the extracellular environment via GPCRs and within the intracellular milieu via the HXK or SnRK1 pathways. There is evidence of Glc-responsive genes being regulated independently of HXK, as well as regulation at the post-transcriptional / translational level. Although the cytosolic Suc and hexose pools are linked, genes specifically regulated by Suc have been identified. Suc was found to repress the *bZIP11* transcription factor at the post-transcriptional level. Many sugar responsive cis-acting elements have been identified in the promoters of transcription factor genes with considerable inter-regulation with plant hormones.

### 1.2.1 Short-term responses to low light

Fixed at one location and reliant on sunlight for energy, plants must adapt to changes in environmental conditions *in situ*. Changes in light flux, especially low light, is one of the most severe threats plants can experience (Franklin & Quail, 2009). The stress of shading or prolonged darkness will naturally cause a large decline in photosynthetic output, requiring the plant to rely on carbohydrate reserves which become rapidly exhausted (Fujiki et al., 2000; Gibon et al., 2004). The name given to the strategy and the processes involved, used by plants to maintain their access to light, is the shade avoidance response (Franklin, 2008).

#### ***Sensing shade***

As explored in the first section of this chapter, plants have sophisticated mechanisms for perceiving the quality, quantity, periodicity and direction of light. These stimuli are sensed and the signals integrated to coordinate plant growth and development appropriately for the prevailing environmental conditions. Plants are able to detect shading via the quality and quantity of light that they receive (Smith, 1982). Phytochromes are reversibly converted between the Pr and Pfr forms by R and FR respectively. The abundance of either form therefore, allows plants to assess the R:FR (eq. 1.2.1.1) of the incident light.

$$R:FR = \frac{\text{photon fluence at } 660\text{nm} \pm 5\text{nm} (R)}{\text{photon fluence at } 730\text{nm} \pm 5\text{nm} (FR)}$$

#### **Equation 1.2.1.1 Equation for ratio of Red to Far-red light flux**

Reproduced from (Smith, 1982).

The R:FR will therefore vary according to the time of day, the site aspect and latitude and greatly on the surrounding plant canopy structure. The R:FR is influenced little by weather conditions or the season (Table 1.2.1.1) (Franklin, 2008; Smith, 1982). It is evident that wavelength is crucial in determining the R:FR; moonlight has a tiny fraction of daylight flux, but there is only a small reduction in its R:FR (Table 1.2.1.1). The strikingly obvious property of the ivy canopy light parameters, is the very low R:FR; even under 5mm of soil, which has about half the flux of ivy canopy, has a much higher R:FR (Table 1.2.1.1). The unique property of plant canopies of course, is their filtering of the blue and R portions of the light spectrum due to the absorption spectra of photosynthetic pigments (carotenoids and chlorophyll respectively). Leaves reflect and

transmit the remainder, which in the visible spectrum gives them their green appearance. However they also leave FR unabsorbed, so underneath a canopy, not only is the light flux much reduced, it is depleted in R and blue light – the photosynthetically active radiation (PAR), resulting in a low R:FR (Franklin, 2008; Franklin & Quail, 2009).

**Table 1.2.1.1 Light parameters of environmental conditions.**

Reproduced from Table 1 in (Smith, 1982).

	Photon Flux Density ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	R:FR
Daylight	1900	1.19
Sunset	26.5	0.96
Moonlight	0.005	0.94
Ivy canopy	17.7	0.13
Lakes, depth 1m	-	-
Black Loch	680	17.2
Loch Leven	300	3.1
Loch Borrallie	1200	1.2
Soil, depth 5mm	8.6	0.88

A decline in R:FR is due to the depletion of PAR most often caused by the shade generated by surrounding vegetation. Without the PAR component of light, plants will be unable to maintain effective photosynthetic output, therefore they must initiate strategies that allow them to tolerate or escape shading; the latter of these two is known as the shade avoidance syndrome (Smith & Whitelam, 1997) or response (Franklin, 2008). As established above, there is a crucial difference between shade characterised by low photo flux and canopy shade: the markedly lower R:FR ratio of the latter (Table 1.2.1.1). However a decline in R:FR was not established as the *sine qua non* for the initiation of shade avoidance syndrome until work carried out by (Morgan & Smith, 1976; Morgan & Smith, 1978; Morgan & Smith, 1981; Smith & Whitelam, 1997). Given that other plants are the source of shading to which plants are responsive, ecology regards shade avoidance as a response to competition for resources – specifically light (Ballaré et al., 1997).

### **Shade avoidance**

In order to be effective, shade avoidance is a pre-emptive strategy in response to a decline in R:FR prior to actual shading (Ballaré et al., 1990). The shade avoidance syndrome can be summarised (Table 1.2.1.2) as shoot elongation and arrested leaf development with a strengthening of apical dominance. In dicotyledonous plants, such as *A. thaliana*, shoot extension is achieved by increases in the length of internodes on

the stem and elongation of leaf petioles. The strengthening of apical dominance ensures that resources are focussed on increasing vertical height of the plant and also promotes the increase in leaf angle (hyponasty). There is a reduction in chlorophyll synthesis, visible as chlorosis (Smith & Whitelam, 1997). This accelerated apical growth requires a reallocation of resources away from the storage organs (Smith & Whitelam, 1997). There is an accumulation of hexose sugars in the leaves and petioles of plants grown in white light enriched with FR (Keiller & Smith, 1989). RNA interference (RNAi) of potato *sucrose transporter 1* (*StSUT1*) inhibits development of shade avoidance syndrome (Chincinska et al., 2007). Low R:FR promotes early onset of flowering in wild-type (Halliday et al., 1994) and this phenomenon is not lost, but only reduced in *phyABD* triple mutants, which are early flowering in high R:FR (Devlin et al., 1996). This suggested phytochrome redundancy and a role for an additional phytochrome, viz. phyC; furthermore evinced by the *phyABDE* quadruple mutants lacking leaf elongation or accelerated flowering in response to low R:FR (Franklin et al., 2003). Resources will need to be reallocated to support flowering, so the onset of senescence (Levey & Wingler, 2005) and autophagy (Hanaoka et al., 2002) in leaves can remobilise nutrients from leaves to support reproduction (Himmelblau & Amasino, 2001).

**Table 1.2.1.2 Physiological processes characterising shade avoidance syndrome**

Reproduced from Table 1 in (Smith & Whitelam, 1997)

<b>Physiological process</b>	<b>Shade response (low R:FR)</b>
<b>Germination</b>	<b>Retarded</b>
<b>Extension growth</b>	<b>Accelerated</b>
Internode extension	Rapidly increased (lag c. 5 min)
Petiole extension	Rapidly increase
Leaf extension	Increased in cereals
<b>Leaf development</b>	<b>Retarded</b>
Leaf area growth	Marginally reduced
Leaf thickness	Reduced
<b>Chloroplast development</b>	<b>Retarded</b>
Chlorophyll synthesis	Reduced
Chlorophyll <i>a:b</i> ratio	Balance changed
<b>Apical dominance</b>	<b>Strengthened</b>
Branching	Inhibited
Tillering (in cereals and grasses)	Inhibited
<b>Flowering</b>	<b>Accelerated</b>
Rate of flowering	Markedly increased
Seed set	Severe reduction
Fruit development	Truncated
<b>Assimilate distribution</b>	<b>Marked change</b>
Storage organ deposition	Severe reduction

## ***Molecular regulation of shade avoidance***

### **PIF/PIL-mediated shade avoidance**

The first genes identified whose expression was found to be reversibly inducible by R:FR was the *A. thaliana homeobox 2* and *4* (*ATHB2,4*). *ATHB2* and *ATHB4* are homeodomain ZIP transcription factors whose expression is induced by low R:FR and repressed under high R:FR conditions (Carabelli et al., 1996; Carabelli et al., 1993). *phyABDE* quadruple mutants, as mentioned above, are not responsive to low R:FR conditions. Dark-adapted *phyABDE* mutant plants demonstrate repression of *ATHB2* expression upon transfer to white light, suggesting a role for phyC in mediating R-dependent repression of *ATHB2* and shade avoidance responses (Franklin et al., 2003). The largest and most rapid increases in transcript levels, following low R:FR treatment, are seen for *PIF3-like 1* (*PIL1*): ~35-fold increase after one hour. *PIL1* encodes a bHLH protein that interacts with the circadian clock central oscillator component, TOC1. Mutants of *PIL1* exhibited reduced hypocotyl elongation following transient R:FR exposure (Salter et al., 2003). PIF4 and PIF5 (phytochrome-interacting factor) promote the shade avoidance response and constitutive expression of either *PIF4* or *PIF5* results in plants displaying the shade avoid response constitutively. *phyB* null mutants demonstrate a constitutive shade-avoidance phenotype under high R:FR, however in *phyBpif4pif5* triple mutants this phenotype is not seen (Lorrain et al., 2008). The abundance of PIF3 rapidly declines in response to light, either FR-mediated by phyA as part of phytochrome-mediated de-etiolation, or by R-mediated by phyB in the Pfr form (Bauer et al., 2004). This decline in abundance is mediated by the ubiquitin-26S proteasome system, whereby PIF3 is polyubiquitinated, mediated by phytochrome Pfr, and then degraded by the 26S proteasome (Park et al., 2004). As with PIF3, PIF4 and PIF5 abundance is light dependent, they accumulate in the dark and decline upon exposure to R; FR did not have a significant effect on their abundance (Lorrain et al., 2008). PIF4 binds preferentially to the Pfr form of phyB in the nucleus. However PIF4 does not interact with phyB when bound to a G-box (Huq & Quail, 2002). The swift decline in PIF4 and PIF5 abundance following a two-hour R pulse to etiolated seedlings, which were returned to darkness, suggested that effect was mediated by the phytochrome low-fluence response. PIF4 and PIF5 are responsive to high and low R:FR treatments; high R:FR decreases the abundance of both PIFs, and low R:FR results in their rapid re-accumulation. As with PIF3, the degradation of PIF4 and PIF5 is proteasome mediated; treatment of etiolated seedlings with proteasome inhibitors and R light sees no decline in the abundance of either PIF (Lorrain et al., 2008).

### **DELLA mediated shade avoidance**

There is further redundancy in the shade avoidance regulation, since *pif4pif5* double mutants still exhibit shade avoidance responses (Lorrain et al., 2008; Franklin, 2008). Implicated are nuclear-localised DELLA proteins, which are known to down-regulate growth by down-regulating gibberellic acid (GA) biosynthesis. External cues cause the activation of GA that represses DELLA genes and so increasing the levels of GA and promoting growth (Silverstone et al., 2001). GA mediates the phosphorylation of the DELLAs which are in turn polyubiquitinated and targeted for degradation by the 26S proteasome (Alvey & Harberd, 2005). Treatment with low R:FR results in a petiole elongation, concomitant with this is a decline in the abundance of DELLA proteins. In GA deficient mutants there is no petiole elongation, but these can be 'rescued' by addition of GA (Djakovic-Petrovic et al., 2007). Whilst the degradation of DELLAs is R:FR-dependent and therefore phytochrome mediated, there is no evidence to suggest a direct interaction between DELLAs and phytochromes. As described above, the extent and manner of the interaction between phytochromes and PIFs has been elucidated (Bauer et al., 2004; Park et al., 2004). Co-immunoprecipitation, yeast-two-hybrid, pull-down and chromatin immunoprecipitation (ChIP) studies confirmed that there is a direct interaction between DELLA protein, RGA, and individual PIF4 (de Lucas et al., 2008) and PIF3 (Feng et al., 2008), whereby under high R:FR DELLAs bind to individual PIFs and block their transcriptional activity (de Lucas et al., 2008; Feng et al., 2008). Quintuple mutants of the DELLAs have a phenotype resembling wild-type treated with GA, underlining the GA-dependent abundance of DELLA proteins. Nuclear localised GA receptors, GID1 proteins, were found to interact with DELLAs. The co-immunoprecipitated DELLAs could be bound by anti-ubiquitin antibodies (Feng et al., 2008). These taken together (Feng et al., 2008) conclude, suggest that GA, whose biosynthesis is promoted by light, binds GID1 which in turn binds DELLAs targeting them for degradation via the ubiquitin-26S proteasome system.

### **Temperature cross-talk in shade avoidance**

Temperature has been found to modulate light-responses; early flowering mutant *phyB*, no longer exhibits its phenotype when the temperature is reduced from 22 to 16°C. The role of *phyB* at lower temperatures becomes redundant as *phyD* and *phyE* also contribute (Halliday & Whitelam, 2003). The expression of *cold regulated (COR)* genes could be induced in seedlings treated with low R:FR at 16°C, but not 22°C (Franklin & Whitelam, 2007). The *COR* genes have C-repeat/drought responsive (CRT/DRE) elements in their promoters, conferring the responsiveness to drought and low

temperature and to this cis-element binds, the CRT/DRE binding-factor (CBF) family transcription factors (Knight et al., 2004). Overexpression of *CBF* genes confers improved freezing tolerance in the absence of cold acclimation (Jaglo-Ottosen et al., 1998), similarly plants grown at 16°C under low R:FR leads to 87% survival versus 38% and severe damage for those grown high R:FR at 16°C. *phyD* mutants grown at 16°C have elevated *COR* gene expression and improved freezing tolerance (Franklin & Whitelam, 2007). There is extensive phenotypic plasticity in response to temperature: at 16°C plants have a small, compact rosette; at 22°C the leaf area and growth rate is at its highest; growth at 28°C results in petiole elongation, leaf hyponasty and reduced leaf area – a phenotype similar to the shade avoidance response (Atkin et al., 2006; Franklin, 2009). Mutants of an ancillary auxin biosynthesis pathway exhibit defective shade avoidance responses (Tao et al., 2008), similar mutants for auxin transport or response pathways exhibit impaired high temperature hypocotyl elongation in seedlings (Gray et al., 1998). High temperature combined with low R:FR had a synergistic effect on the shade avoidance response (Weinig, 2000); these findings point to shared signalling pathways (crosstalk) between light and temperature (Franklin, 2009).

### ***Carbon scavenging and sugar signalling***

#### **Physiological responses to dark-induced sugar starvation**

Plants, as we have seen, are able to trigger processes that facilitate their overcoming of shade, however those processes require resources. In the light photosynthesis takes place, providing the plants with a source of energy in the form of fixed carbon. Sucrose synthesis takes place in source tissues so supports growth in sink tissues. During darkness photosynthesis cannot proceed and so plants are reliant on their reserves of energy in the form of starch (Geiger & Servaites, 1994; Gibon et al., 2004). Plants are able to perceive the day length and store up sufficient starch to support respiration during the night (Gibon et al., 2004). Starch synthesis is under circadian control – being initiated one to two hours into the light phase and slowing about two hours before darkness. A circadian rhythm of starch synthesis is seen even with plants kept in constant light (Geiger & Servaites, 1994). By the start of the next light cycle very little starch remains (Kerr et al., 1985; Fondy & Geiger, 1985; Gibon et al., 2004). Indeed plants exposed to extended darkness exhibited a dramatic decline in soluble sugars, far beyond that seen with diurnal fluctuations (Kerr et al., 1985; Brouquisse et al., 1998). A significant decrease in protein levels was also seen in mature root, root tips and young leaves. There are no significant diurnal fluctuations in proteolysis, but during extended

night protein degradation was induced, concomitant with a change in the glutamine (Gln) / asparagine (Asn) balance, whereby levels of free Gln dropped with Asn abundance increasing up to 18 times in mature roots (Brouquisse et al., 1998). Proteolysis will liberate significant amounts of toxic  $\text{NH}_4^+$ , which is assimilated by the conversion of aspartate (Asp) to Asn. Asn synthesis is preferred to Gln synthesis under carbon limiting conditions such as extended darkness (Lam et al., 1996; Brouquisse et al., 1998). Studies using mutants for the gene coding phosphoglucomutase (*pgm*), which are unable to convert G6P to glucose 1-phosphate (G1P) and hence are unable to synthesise starch, revealed that with a 12-hour photoperiod *pgm* mutants accumulated high levels of soluble sugar compared to wild-type and their phenotype and growth was indistinguishable from wild-type under constant light. However when grown under short-day (seven-hour photoperiod) conditions the *pgm* growth rate was severely impaired and photosynthetic rate depressed compared to wild-type (Caspar et al., 1985). Protein degradation, of up to 45% of original intracellular protein, was induced in tobacco (*Nicotiana tabacum*) suspension-cultured cells upon transfer from liquid Murashige-Skoog (MS) medium containing 3% (w/v) Suc to MS without Suc (Moriyasu & Ohsumi, 1996). The large scale degradation of protein triggered by sugar starvation / extended darkness (Brouquisse et al., 1998) is known as autophagy and is mediated by the vacuolar degradation pathways (Hanaoka et al., 2002). Sugar starvation of cultured sycamore (*Acer pseudoplatanus L.*) cells resulted in the formation of double membrane-bound autophagosomes (vacuoles) which fused with the central vacuole. The initiation of autophagy was found to dependent of supply of pyruvate or glycerol to the mitochondria, independent of Suc or hexose sensing (Aubert et al., 1996). 25 genes in *A. thaliana* have been identified as orthologues to nine yeast genes essential for autophagy. Mutants for these genes in yeast were found to exhibit impaired protein degradation, reduced survival under sugar starvation conditions and defective diploid cell sporulation. The *A. thaliana* *apg9-1* mutant, orthologue of yeast *APG9*, exhibited accelerated chlorosis (leaf yellowing) compared to wild-type after eight days of 24-hour darkness. *apg9-1* mutants demonstrated elevated expression of senescence associated genes, *SENI (DINI)* and *YSL4*, prior to the induction of senescence, unlike wild-type (Hanaoka et al., 2002). (Yu, 1999) summarised the physiological changes in response to sugar-starvation as follows: arrest of cell growth; rapid depletion of carbohydrates; decrease in respiration rate; degradation of proteins and lipids, accumulation of inorganic phosphate ( $\text{P}_i$ ), phosphorylcholin, free amino acids and a decline in glycolysis. The reduction in biosynthetic processes and respiration will help to conserve

resources and the induction of catabolic processes will liberate alternative sources of carbon from lipids, proteins and structural carbohydrates (Yu, 1999).

### **Mediation of sugar starvation responses**

A whole-genome expression profile array, which analysed the responses to six-hour extended night, having been grown in a 14-hour photoperiod, allow a comparison between wild-type and *p<sub>g</sub>m* mutant expression patterns at the end of the night period, revealed similarities between both genotypes: there was repression of genes involved in photosynthesis, nutrient acquisition and biosynthesis; changes in Tre metabolism suggested a role for T6P signalling sugar-starvation; alterations in transcript levels of receptor kinases, transcription factors and signalling pathways; changes to genes controlling protein turnover and post-translational modifications; modifications to the genes involved in the synthesis and signalling pathways of plant hormones such as cytokinins, ABA and ethylene (Thimm et al., 2004). The Thimm (2004) study has identified a complex, multi-level regulatory network in mediate responses to low sugar availability.

The transcripts of two genes, coding for the E1 $\beta$ - and E2-subunits of branched-chain  $\alpha$ -ketoacid dehydrogenase (BCKDH) of *A. thaliana*, henceforth *DIN4* and *DIN3* respectively, were found to accumulate in leaves after three hours of darkness, with a concomitant increase in BCKDH activity. The induction of *DIN3/4* expression could be replicated by the application of a photosynthesis inhibitor (DCMU) even with illumination. Furthermore it was discovered that the induction of *DIN3/4* was repressed by supplementation with Suc but not Man, thus indicating that *DIN3/4* is regulated primarily by carbon status, rather than directly by light (Fujiki et al., 2000). Fujiki et al., (2001) identified a number of other *DIN* genes, reporting that they code for a variety of functionally diverse proteins: *DIN1* (*SENI*) is a senescence-associated gene with an unknown role (Schenk et al., 2005); *DIN2* encodes a protein with strong similarity to  $\beta$ -glycosidases involved in cell wall degradation; *DIN6* is identical to *ASNI* that encodes asparagine synthetase (AS); *DIN9* encodes a protein with homology to mannose synthesis enzyme, phosphomannose isomerase (PMI); *DIN10* encodes a protein homologous to seed imbibition protein, both of which share similarities with stachyose and raffinose synthases – enzymes that produce oligosaccharides which accumulate during response to senescence (Fialho & Bucker, 1996), drought and low temperatures (Bachmann et al., 1994); *DIN11* results in a protein similar to dioxygenases, which are active in senescent leaves (Woodson et al., 1992), involved in the biosynthesis of GA,

ethylene, flavinoids and alkaloids (Prescott & John, 1996). (Fujiki et al., 2005) divide the *DIN* genes into two groups: early responders and late responders. The early responders include *DIN3*, *4*, *6* and *10* whose transcripts accumulate within three hours of a dark treatment, peaking between 24 – 48 hours, and the late responders, including *DIN2*, *9*, and *11*, detected only after 24 hours and continuing to accumulate after 72 hours (Fujiki et al., 2000; Fujiki et al., 2001). This dichotomy in transcript kinetics is also reflected in developmental transcript abundance. Levels of late-responsive *DIN2* and *DIN9* were accumulated during darkness more strongly in old leaves than younger leaves and in early senescence mutant *hys1*; conversely the abundance of early-responsive *DIN3* was independent of leaf age or accumulated more strongly in younger leaves (Fujiki et al., 2005).

### **Dark-inducible gene regulation**

Further experiments using Glc analogues 2-DG and 3-OMG, revealed that 2-DG, which can be phosphorylated by HXK but not metabolised, was able to suppress *DIN* gene expression, whereas 3-OMG, which is not phosphorylated by HXK, could not. Additionally, application of a Ser/Thr protein kinase inhibitor, K-252a, prevented the induction of *DIN* genes following dark treatment, as did protein synthesis inhibitor, cycloheximide (Fujiki et al., 2000). SnRK1 (discussed in detail in §1.1.3) is such a Ser/Thr kinase (Smeekens, 2000), which under sugar-starvation conditions has been shown to be a positive regulator of genes involved in starch and protein catabolism (Baena-González et al., 2007), and so may be involved in regulating *DIN* genes. Use of two protein phosphatase inhibitors, okadaic acid, which preferentially inhibits protein phosphatase type 2A (PP2A), and calyculin A, which is more potent and inhibits both PP1 and PP2, revealed a role for protein dephosphorylation in regulating sugar-induced gene expression (Fujiki et al., 2000). Okadaic acid application caused enhancement of *DIN* gene transcripts in sugar-starved cells except for *DIN6* and *DIN10*. Calyculin A had the precisely the reverse effect, inhibiting *DIN* gene transcripts in sugar-starved cells, but not *DIN2* and *DIN9* transcripts. These results suggested that PP2A, which is preferentially inhibited by okadaic acid, has an inhibitory role on most *DIN* genes, whilst calyculin A sensitive PP1 is required for sugar-induced expression of *DIN* genes; therefore protein phosphorylation events are involved in sugar-responsive gene expression (Fujiki et al., 2000). Hence Fujiki et al., (2000) divide the *DIN* genes into two groups, the first consisting of *DIN1*, *3* and *4*, which are negatively regulated by PP2A and positively by PP1. The second group: *DIN6* and *10*, insensitive to PP2A and positively regulated by PP1. Then a third: *DIN2* and *DIN9*, negatively regulated by both

protein phosphatase types (Fujiki et al., 2000). Use of calmodulin inhibitor W-7 caused increased dark-induced accumulation of *DIN2* and *DIN9* transcripts, whereas no effect was seen with *DIN3* transcripts. This suggests that  $\text{Ca}^{2+}$ /calmodulin signalling regulates the late-responsive, senescence-associated *DIN2* and *DIN9*, negatively in darkness. Slight enhanced dark-induced expression of *DIN3* was seen with two other calmodulin inhibitors, which may indicated a role for  $\text{Ca}^{2+}$ /calmodulin mediated repression of *DIN3* (Fujiki et al., 2005). However (Fujiki et al., 2005) found no putative cis-acting elements for calmodulin binding proteins, senescence responsive elements or conserved novel motifs in the promoters of *DIN2* and *DIN9*.

### **Summary**

Using information from the quantity and quality of incident light borne out in the R:FR and the resultant Pr/Pfr equilibrium, plants are able to perceive shading. Plants undergo various physiological changes in response to shading, which are collectively called the shade avoidance response. This response is mediated by phytochromes and the differential localisation between the two forms. Phytochromes and interact with PIF/PIL proteins and PIFs with DELLA proteins to bring about the swift physiological changes required. Light and temperature signalling are integrated into common regulatory pathways, such that low R:FR at 16°C can initiate increases in *COR* gene expression and high temperature can produce phenotypes resembling the shade avoidance response, which have the same reliance on auxin signalling. Insufficient light from deep shade or complete darkness outside the normal diurnal cycle results in acute sugar starvation. Depletion of carbohydrates combined with the cessation of photosynthesis constitutes a severe energy crisis for a plant, which results in genome-wide changes in gene expression to conserve energy by reducing carbon consuming processes and liberate energy from alternative carbon sources. Studies of the regulation of *dark inducible* (*DIN*) genes have revealed that the mediation of the response to sugar starvation is multifarious: requiring functioning protein kinases, which may include HXK and SnRK1, (de-)phosphorylation events as well as indicating a role for  $\text{Ca}^{2+}$ /calmodulin signalling pathways.

### **Project aims**

It has been the aim of this project to investigate the contribution made by cis- and trans-acting regulatory elements to the responsiveness of *DIN3* expression to environmental stimuli: specifically darkness and chilling. Continuing previous work (Knight, H., unpublished), which revealed the minimal dark-responsive promoter sequence of *DIN3*,

and via linker-scan mutation analysis primarily, the contribution of cis-acting factors within a crucial 50 base-pair (bp) region of the promoter were investigated. Secondly, through the creation and analysis of transgenic plant lines over-expressing transcription factor genes, candidates for trans-acting factors regulating *DIN3* were analysed. Following the Chapter 2 – Materials and Methods, the results are divided up in the former manner with consideration of the cis-acting factors in Chapter 3 followed by the trans-acting factors in Chapter 4. The results in their entirety will be discussed in Chapter 5.

## **2 Materials and Methods**

### **2.1 Materials**

#### **2.1.1 Chemicals**

All chemicals used in this project were obtained from *Sigma-Aldrich Company Ltd.* (Dorset, England) unless stated otherwise in the method. Antibiotics for selection media used in the culture of transformed *E. coli* or *A. tumefaciens* were all sourced from *Melford Laboratories Ltd.* (Ipswich, UK).

#### **2.1.2 Plant Materials**

The wild type *Arabidopsis thaliana* plants used in the experiments described in this work were of the Columbia ecotype, accession 0 (Col-0) unless otherwise stated. All wild type plants were grown from seeds originally obtained from *Lehle Seeds* (Round Rock, USA). Plants over-expressing various transcription factor genes were transformed in the laboratory. Plants of various TILLING lines were originally obtained from the *Seattle TILLING Project* (<http://tilling.fhcrc.org/>) and the T-DNA insertion lines were grown from seeds originally obtained from *GABI – Genomanalyse im biologischen System Pflanze* (Max-Planck Institut für Molekulare Pflanzenphysiologie, Potsdam, Germany). Plants of the 12 linker scan lines were available in the lab at the start of this project.

#### **2.1.3 Bacterial Strains**

*Escherichia coli* competent cells were DH5 $\alpha$  cells (*Bioline Ltd.*, London, UK) and transformed using plasmids previously designed. Chemically competent cells were made in the lab from the *Agrobacterium tumefaciens* strain C58C1.

#### **2.1.4 Enzymes for Nucleic Acid Modification**

Enzymes used in the modification or treatment of nucleic acids were sourced from a variety of suppliers. Taq-DNA polymerase (BIOTAQ™ Red DNA Polymerase) used in regular PCR reactions was sourced from *Bioline UK Ltd.* (London, UK). Reverse Transcriptase (MultiScribe™ Reverse Transcriptase) for random-primed (random hexamers) cDNA synthesis from RNA was obtained from *Applied Biosystems* (Foster City, USA). Full-length cDNA transcription from RNA was carried out using *Epicentre MMLV-RT 1<sup>st</sup> Strand cDNA Synthesis Kit* (*EPICENTRE Biotechnologies*, Madison, USA). The reaction mix including the enzyme for Real-Time PCR using Taqman® probes (TaqMan® Gene Expression Master Mix) was also obtained from *Applied*

*Biosystems* (Foster City, USA). The reaction mix for qRT-PCR carried out using SYBR® Green with primers (SensiMix SYBR Kit) was also obtained from *Bioline UK Ltd.*

### **2.1.5 Oligonucleotides**

All oligonucleotides for primers for PCR and qRT-PCR were synthesised by *Invitrogen Ltd.* (Paisley, UK).

## **2.2 Preparation of Biological Materials**

### **2.2.1 Sterilisation Techniques**

#### **2.2.1.1 Solutions and Media**

Solutions and media, whose components are tolerant of the high temperatures and pressures, were sterilised using an autoclave set to deliver a temperature of 121°C at a pressure of  $1.03 \times 10^5$  Pa for 20 minutes. Those solutions and media not tolerant to autoclave conditions were filter sterilised using 0.2µm cellulose acetate sterile syringe filters, sourced from *VWR International Ltd.* (Leicestershire, UK), and attached to a 10ml syringe obtained from *BD* (Oxford, UK).

### **2.2.2 Growth Media**

#### **2.2.2.1 Plant Growth Media**

##### **2.2.2.1.1 Standard Petri Plate Media**

*A. thaliana* plants used in the majority of experiments of this work were sown as seeds onto solid agar media (Murashige & Skoog, 1962) in Petri dishes. Triple vent Petri dishes of 90 (*Scientific Laboratory Supplied Ltd.*, Hessle, UK) and 55mm (*Fisher Scientific UK Ltd.*, Loughborough, UK) were used with solid agar based media. The media consisted of 0.8% (w/v) plant cell culture tested agar (*Sigma-Aldrich Company Ltd.*, Dorset, UK) with 1 x Murashige and Skoog (MS) nutrient medium (*Duchefa Biochemie*, Ipswich, UK) with a pH of 5.8 set using 0.1M Potassium Hydroxide (KOH). Sterile autoclaved media was poured into the Petri dishes in a laminar flow hood once hand-hot (~50°C). The lids were replaced whilst the media set to prevent changes in the relative concentration of the components of the media due to evaporation.

##### **2.2.2.1.2 Vertical Petri Plate Media**

*A. thaliana* plants grown in the vertical position, as opposite to the standard horizontal orientation, were grown on 120mm square Petri dishes (*VWR International Ltd.*,

Lutterworth, UK) with solid agar media composed of 1.2% (w/v) plant cell culture test agar with 1x MS and adjusted to pH 5.8. The medium was poured within a laminar flow hood. Seedlings were transplanted to this medium after 7 days of being grown on the standard Petri plate media in the horizontal position.

### **2.2.2.1.3 Selection Media**

Plant media for the selection of primary transformant plant requires the addition of chemicals to eliminate those plants, which do not carry the transgene. The sole plant selection media used in this project was composed of 0.8% (w/v) plant cell culture tested agar, with 1x MS, 1% (w/v) Sucrose (SUC) with Kanamycin to a concentration of 50µg/ml and adjusted to a pH of 5.8. The Kanamycin antibiotic was applied to the autoclaved medium once hand-hot within a laminar flow hood. The complete medium was poured into circular 140mm triple vent Petri dishes.

### **2.2.2.2 Bacterial Growth Media**

#### **2.2.2.2.1 E. coli Media**

The liquid media used for the culture of *E. coli* was composed of Luria-Bertani (LB) broth with the addition of an antibiotic selection, if required. The only antibiotic used in regard to the culture of *E. coli* in this project, was Spectinomycin at a concentration of 50µg/ml. *E. coli* were also cultured on 90mm Petri dishes filled with a medium composed of 1.5% (w/v) agar, 2% (w/v) LB and with Spectinomycin (50µg/ml) applied once the autoclaved media had cooled to hand-hot within a laminar flow hood. The media was poured into the plates within the laminar flow hood and allowed to set with the lids off. The long term storage of bacterial cultures was achieved using 0.5ml 50% (v/v) Glycerol and 0.5ml of the bacterial culture, which was flash frozen in liquid nitrogen and stored at -80°C.

#### **2.2.2.2.2 A. tumefaciens Media**

The media used for the culture of *A. tumefaciens* was identical in composition for those used for *E. coli* (2.2.2.2.1 *E. coli* Media) except that the antibiotic used in all *A. tumefaciens* media was Rifampicin at a concentration of 100µg/ml. The C58C1 strain of *A. tumefaciens* contains a resistance gene to Rifampicin on its chromosomal plasmid. Additional antibiotics were required to select for *A. tumefaciens* transformed by binary plasmids which contained antibiotic resistance genes.

## **2.3 Culture of Biological Materials**

### **2.3.1 Sterilisation of *A. thaliana* seeds**

#### **2.3.1.1 Ethanol Sterilisation Technique**

Sterilisation of seeds, indicated to have low levels of contamination from mould or fungi, was carried out using the Ethanol technique whereby the required amount of seeds was placed into a 1.5ml microcentrifuge tube to which was added 70% (v/v) Ethanol. The tubes of seeds were then shaken on a low speeding using a bench-top *Labnet* (Oakham, UK) vortex with the necessary attachment for 10 minutes. The seeds were then removed from the tubes via pipette onto 90mm filter circles (*Whatman Plc.*, Maidstone, UK) to dry within a laminar flow hood prior to sowing onto the appropriate medium.

#### **2.3.1.2 Bleach Sterilisation Technique**

Sterilisation of seeds, indicated to have high levels of contamination from mould or fungi, was carried using the bleach technique. This technique was also used when harvesting the seeds of plants dipped with *A. tumefaciens*. The bleach method was most often used to sterilise large quantities of seeds so carried out using 50-ml *Fisher* (*Fischer Scientific*, Loughborough, UK) centrifuge tubes. Along with the seeds, 70% (v/v) Ethanol was added to almost fill the centrifuge tube and agitated on at 300 rpm on a flat-bed shaker for 5 minutes. Transferred to a laminar flow hood, the Ethanol was decanted from the tubes and replaced with a solution of 10% (v/v) Sodium Hypochlorite and 0.25% (v/v) Sodium Dodecyl Sulphate (SDS). The tubes were returned to the shaker and agitated at 300 rpm for 10 minutes. Returned to the laminar flow hood, the solution was decanted and the seeds washed six times with sterile MilliQ (MQ) water. The sterilised seeds could then be transferred via a Pasteur pipette to plates of an appropriate medium.

### **2.3.2 Plant tissue culture**

Plants were sterilised using the appropriate technique (2.3.1 Sterilisation of *A. thaliana* seeds) and sown onto the required Petri plate medium within a laminar flow hood and the plate sealed with Micropore™ tape (*3M United Kingdom PLC.*, Bracknell, UK). In some cases seeds were sown directly onto soil, composed of mixture J Arthur Multipurpose Compost and sharp sand, using an adapted 50-ml centrifuge tube filled with baked sand to provide an even distribution of seeds. The lid of the 50-ml centrifuge tube was pierced to create several holes and the seeds mixed well with the baked sand

so to that seeds were widely dispersed across the soil. Once sown onto the medium the seeds were stratified for 48 hours in a cold room set to 5°C. If the plants were to be grown under long-day conditions this was carried out within a *Sanyo MLR-351* plant growth chamber (*Sanyo E&E Europe BV, Biomedical Division (UK)*, Loughborough, UK) set to a 16 hour light/8 hour dark cycle at a continuous temperature of 20°C unless otherwise stated. For plants grown under short-day conditions this meant an 8 hour dark/16 hour light cycle at a continuous temperature of 20°C unless otherwise stated (in the same chamber). The mean light intensity with these growth chambers was 150  $\mu$ Einsteins. Any other conditions for plant tissue culture will be described in figure legends. Three and six hour dark treatments were achieved by wrapping whole Petri plates in aluminium foil and returning then to the plant growth chamber of appropriate temperature (5 or 20°C) for the length of the treatment. Plants used in experiments which required growth beyond 14 days post germination, seedlings were transferred to 38mm peat plugs (*LBS Horticulture Ltd.*, Lancashire, UK) and grown under the conditions stated in the experimental method.

### **2.3.3 Bacterial culture**

#### **2.3.3.1 Small scale plasmid preparation**

Small scale preparation of bacterial cultures was carried out using a 5ml overnight bottle containing 2% (w/v) LB broth and any required antibiotics (Rifamicin 100 $\mu$ g/ml for *A. tumefaciens*). These overnight bottles were inoculated either using a flamed inoculation loop or a sterile pipette tip. The loop or pipette tip was used to touch a colony on plate or scratch the surface of a frozen glycerol stock and then dipped or ejected into the overnight bottle. The aseptic technique of flaming inoculation loops before and after use and the necks of bottle after opening and closing their lids. The inoculation of the overnight bottles was carried out in the immediate vicinity of a lit Bunsen burner. If inoculated with *E. coli* the overnight bottles were placed in an incubator set to 37°C and shaking at 200 rpm and left overnight. If inoculated with *A. tumefaciens* the overnight bottles were placed in an incubator set to 28°C and shaking at 200 rpm and left for 48 hours.

#### **2.2.3.2 Medium scale plasmid preparation**

Medium scale preparations were carried out in baffled 500ml flasks containing 200ml of 2% (w/v) LB broth in addition to any required antibiotics. These flasks were then inoculated with 1ml of starter culture (2.3.3.1 Small scale plasmid preparation).

These flasks were then moved to incubators set the appropriate conditions for the bacterial strain as described in the above section.

#### **2.2.4.3 Petri plate culture**

Bacterial cultures were grown on Petri plates as described above (2.2.2.2 Bacterial Growth Media) and inoculated using either a flamed inoculation loop to streak out a picked colony or glycerol stock, or with 100µl from a starter culture (2.3.3.1 Small scale plasmid preparation) and spread across the surface of the plate using a sterile glass spreader. The inoculation of the plates was carried out in the immediate vicinity of a lit Bunsen burner. If the plates had been inoculated with *E. coli* the plates were transferred to an incubator set to 37°C and left overnight. If the plates were inoculated with *A. tumefaciens* then the plates were transferred to an incubator set to 28°C and left for 48 hours.

### **2.4 Plant transformation**

The transformation of *A. thaliana* requires a number of preparatory steps. The methods are described here in sequence.

#### **2.4.1 Transformation of *E. coli***

The transformation of *E. coli* using plasmid DNA (see below) was achieved using DH5α cells (*Bioline Ltd.*, London, UK) which had been stored at -80°C in 25µl aliquots. These cells were transformed using 2.5µl (10% of their volume) and heat shocked according to the prescribed *Bioline* protocol. The transformed *E. coli* were used to inoculate 5ml overnight bottles for a small scale preparation (2.3.3.1 Small scale plasmid preparation).

#### **2.4.2 Mini-scale Plasmid Preps from *E. coli***

When the plasmid carrying the required transgene needed to be purified from a bacterial culture for diagnostic sequencing or subsequent cloning, a small-scale plasmid prep was carried out using *Qiagen* Qiaprep spin miniprep kits (*Qiagen Ltd.*, Crawley, UK). The plasmid prep was carried out according to the method described in the protocol provided by the manufacturer.

#### **2.4.3 Transformation of *A. tumefaciens***

The transformation of *A. tumefaciens* with the desired plasmid was achieved using the competent strain C58C1. Competent cells were prepared by the following procedure, beginning with streaking cells onto an LB + Rifampicin (100µg/ml) plates and

incubating at 28°C for 48 hours. A single colony from this plate was picked and used to inoculate a 5ml overnight bottle containing LB + Rifampicin (100µg/ml) which was incubated at 28°C and shaking at 200 rpm overnight. 4ml of this starter culture was then used to inoculate a sterile 500ml flask contain 100ml of LB without any antibiotics. The flask was then incubated at 28°C and shaking at 250 rpm until the optical density at 600nm (OD<sub>600</sub>) reached between 0.5 and 1.0, determined by a spectrophotometer. This usually takes between 4 and 8 hours. The culture was then chilled on ice prior to centrifugation at 3000g and at 4°C using a *Beckman Coulter* table top centrifuge model Allegra X-22R with rotor 2402 (*Beckman Coulter UK Ltd.*, High Wycombe, UK). The supernatant was discarded and the pellet resuspended in 2ml of ice-cold 20mM CaCl<sub>2</sub> solution. The cells were then dispensed into pre-chilled 1.5ml microcentrifuge tubes as 100µl aliquots then frozen in liquid nitrogen and stored at -80°C for future use.

A 100µl aliquot of the C58C1 strain of *A. tumefaciens* was thawed on ice then up to 1µg of plasmid in a maximum volume of 12µl was applied to the cells and the microcentrifuge tube was gently inverted a few times to mix them and returned to the ice. Next the cells were heat-shocked by placing the microcentrifuge tube in a 37°C water bath for 5 minutes to stimulate the uptake of the DNA by the competent cells. The cells were then transferred via pipette to a sterile overnight bottle containing 1ml of LB without antibiotics and thence placed in an incubator set to 28°C and shaking an no more than 150 rpm for 1 – 2 hours. The contents of the sterile overnight bottle was then transferred to a fresh microcentrifuge tube and centrifuged for 30 seconds and maximum speed. The majority of the supernatant was discarded, but leaving about 200µl in which to resuspend the cells. The resuspended cells were then transferred and spread across an agar plate containing LB + Rifampicin (100µg/ml) and any other antibiotic required to select for the plasmid (Kanamycin at 100 µg/ml or Spectinomycin at 50µg/ml were used for transformations in this project). The plate was incubated for 3 days at 28°C.

#### **2.4.4 Transformation of *A. thaliana***

The transformation of *A. thaliana* requires planning and careful timing to ensure that both the plants and the *A. tumefaciens* are ready simultaneously. This method is adapted from (Clough & Bent, 1998) who originally described the ‘floral dip’ method of *A. thaliana* transformation. Wild type *A. thaliana* plants of Columbia ecotype, accession 0 were used in all plant transformation in this project. Wild type seeds were sown on

standard Petri plate media and grown for 7 days under long-day conditions. Three seedlings were transplanted to each 42mm peat plug and placed into clear boxes with the lids one for the first two days and removed gradually over a few days. These plants were grown under long-day conditions until the first bolts began to form. These were cut immediately and the process of *A. tumefaciens* transformation (above) was begun. Once single transformed colonies were identified, one was used to inoculate an overnight bottle containing 5ml LB + Rifampicin and the additional antibiotic (100µg/ml) then incubated at 28°C and shaking at 200 rpm for 48 hours. 1ml of this starter culture was then used to inoculate a 500ml flask containing 200ml of LB with the appropriate antibiotics (200ml provides sufficient culture to dip 20 – 30 peat plugs) then incubated at 28°C and shaking at 250 rpm for 24 hours. The remaining starter culture was used to make glycerol stocks (2.2.2.2.1 *E. coli* Media). The 200ml *A. tumefaciens* culture was centrifuged for 15 minutes at 3500g at room temperature and the supernatant discarded. Meanwhile a 5% (w/v) sucrose solution was prepared to a volume between 0.5 and 1 times that of the original culture (i.e. 100 – 200ml). Immediately prior to use 50µl of Vac-in-Stuff (Silwet L-77 surfactant) (*Lehle Seeds*, Round Rock, USA) was added to the sucrose solution in which the pelleted cells were resuspended gently by Pasteur pipette. Some this culture was then transferred to a small beaker and the aerial portion of each plant was dipped into it for a few seconds. The plants were placed horizontally in a large tray lined with tissue paper then covered with cling film or a propagator lid and returned to where they had been growing, overnight. The next day the film or the lids were removed and the plants righted to the vertical orientation. If high efficiency transformation was required a further floral dip was carried out 7 – 14 days after the first.

#### **2.4.5 Selection of transformed seedlings**

After the dipped plants had completed their life cycle and their seeds harvested (2.4.6

Seed collection), transformant plants had to be identified. Two strategies were used in the selection of primary transformants: those transformed using a plasmid conferring Kanamycin resistance were sown as seeds on plant selective media plates containing 1% (w/v) sucrose and Kanamycin to a concentration of 50µg/ml (2.2.2.1.3

Selection Media). These were grown under long-day conditions for 7 – 10 days post germination following an extended 4 day stratification at 5°C. Seedlings which had produced their first set of true leaves and appear healthy were transplanted to 38mm peat plugs and placed in trays (*LBS Horticulture Ltd.*, Lancashire, UK). Alternatively

those transformed using a plasmid conferring Basta (Glufosinate) resistance were sown as seeds directly onto trays of compost (2.3.2 Plant tissue culture), covered with aluminium foil and stratified at 5°C for 5 days, after which the trays were moved to a growth chamber set to long-day conditions. 7 days after the seedlings had germinated they were sprayed with Basta (40mg/l ~200µM) (*Bayer CropScience*, Cambridge, UK) so that they were all covered with a fine mist of the solution. The Basta treatment was repeated at 5 day intervals until resistant seedlings had been identified and could be transplanted to 38mm peat plugs and trays. These primary transformant plants were then grown to produce seeds which were harvested for subsequent confirmation of the presence of transgene via PCR using genomic DNA and transgene expressing via QRT-PCR, prior to use in any experiments.

#### **2.4.6 Seed collection**

Once *A. thaliana* plants had completed their lifecycle and entirely dried out, their seeds were ready for harvest. Plants grown on peat discs next to others of a different genotype were isolated using the *Arasystem* (*BETATECH bvba*, Ghent, Belgium) composed of *Aratrays* to hold the water, *Araflats* to hold the peat discs contained within *Arabaskets*; *Aracons* composed of *bases* and *tubes*, were made of clear plastic through which the aerial portion of the plants grew. Dried plants were cut at their base and their siliques striped from their stems on A3 paper. Using a fine tea strainer the seeds were separated from the chaff and placed into small re-sealable plastic bags. The collected seeds were then placed in a 37°C incubator for 48 hours to dry.

### **2.5 Nucleic Acid Preparation**

#### **2.5.1 DNA extraction**

The extraction of DNA from bacterial plasmids has already been described (2.4.2 Mini-scale Plasmid Preps from *E. coli*) this section will concern extracting genomic DNA from plants and purifying DNA from bands extracted from DNA gel electrophoresis or PCR products.

##### **2.5.1.1 Genomic DNA extraction from plant tissue**

The technique used to extract genomic DNA from plant tissues was modified from that described by (Edwards et al., 1991). As far as possible, two young leaves (~10mm x 5mm) or around 5 7-day-old seedlings were harvested and placed into a sterile 1.5ml microcentrifuge tube. The tissue was ground using a pellet pestle (*Anachem Ltd.*, Luton, UK) for 15 seconds, then 400µl of extraction buffer (Tris HCl 200mM, pH 7.5; NaCl

250mM; EDTA 25mM; 0.5% (v/v) SDS) was added to each microcentrifuge tube and vortexed for 5 seconds. The microcentrifuge tubes were centrifuged at maximum speed for 1 minute and then 300µl of the supernatant transferred to fresh labelled microcentrifuge tubes. To each tube 300µl of propan-2-ol was added and the samples inverted gently twice and left to stand for at least 2 minutes. The samples were centrifuged at maximum speed for 5 minutes and the supernatant discarded. Residual supernatant was removed by tapping the tubes onto tissue paper. Any remaining solvent was allowed to evaporate by leaving the tubes open for about 30 minutes. The pellets in each tube were resuspended in 100µl of 1x TE buffer (10mM Tris HCL, pH 8; 1mM EDTA) and left to fully dissolve overnight at 5°C before being stored at -20°C.

#### **2.5.1.2 Purifying DNA from electrophoresis gels**

DNA isolated and subsequently separated by gel electrophoresis can be extracted for further manipulation such as sequencing or cloning. Isolated DNA appeared as bands on gel pre-stained with Ethidium Bromide (EtBr) (10mg/ml) when visualised on a UV transilluminator (*Uvitec Ltd.*, Cambridge, UK). To extract the isolated DNA the band were excised using a scalpel and placed into a sterile 1.5ml microcentrifuge tube and purified according the protocol provided with the *Qiagen* MinElute Gel Extraction Kit (*Qiagen Ltd.*, Crawley, UK).

#### **2.5.1.3 Purifying DNA PCR products from reaction mixes**

In a similar fashion amplified DNA from Polymerase Chain rection (PCR) could be purified directly from the PCR reaction mix. The entire mixture was treated according to the protocol provided with the *Qiagen* Qiaquick PCR Purification Kit in order to extract the purified PCR product (*Qiagen Ltd.*, Crawley, UK).

### **2.5.2 RNA extraction**

The extraction of RNA from plant tissue was always carried out using ~25 7-day-old *A. thaliana* seedlings and following the technique described in the method supplied the *Qiagen* RNeasy MiniKit (*Qiagen Ltd.*, Crawley, UK).

## **2.6 Nucleic Acid Analyses**

### **2.6.1 DNA Gel Electrophoresis**

All DNA in this project was visualised by gel electrophoresis on gels of the following composition unless otherwise stated in figure legends: 1% (w/v) agarose in 0.5x TBE buffer, pre-stained with EtBr (10mg/ml) to a final concentration of 5µg/ml. The EtBr

was added to the molten gel once hand-hot, mixed and then the gel poured into the mould with the appropriate gel combs. All DNA visualised by gel electrophoresis in this project were products of PCR using *Bioline* BIOTAQ Red DNA Polymerase (*Bioline Ltd.*, London, UK) which already contained loading buffer. The size ladder used in all gel electrophoresis to approximate the fragment size was *Bioline* HyperLadder™ I (*Bioline Ltd.*, London, UK).

## **2.6.2 Nucleic Acid quantification**

The concentration of purified DNA and RNA samples were determined using a *Nanodrop* Spectrophotometer model ND-100 (*Nanodrop Products*, Delaware, USA). 2µl of undiluted sample was loaded on the spectrophotometer pedestal and the reading recorded.

## **2.6.3 Polymerase Chain Reaction (PCR)**

### **2.6.3.1 Reaction mix composition**

*Bioline* BIOTAQ Red DNA Polymerase (*Bioline Ltd.*, London, UK) was used for all PCR carried out in this project. Supplied with *Bioline* BIOTAQ Red DNA Polymerase, were 10x NH<sub>4</sub><sup>+</sup> reaction buffer and MgCl<sub>2</sub> (50mM). A dNTPs were supplied by *Bioline* (*Bioline Ltd.*, London, UK) these together with the 10x buffer and MgCl<sub>2</sub> were used at final concentrations of 1.5mM and 10µM respectively as recommended by the *Bioline* protocol. Primers were used from stocks at 50µM or 100µM to give a final concentration of 1mM in the reaction. Reactions had total volumes of 25 or 50µl.

### **2.6.3.2 PCR conditions**

All PCRs were carried out in one of either three thermal cycler models: Px2 Thermal Cycler (*Thermo Fisher Scientific*, Waltham, Massachusetts, USA), *Hybaid* Omn-E or PCR Express (*Thermo Hybaid*, Ashford, UK). The typical PCR conditions are outlined in Table 2.6.3.2.1, with only the number of cycles and annealing temperature, which is dependent on the melting temperature of the primer pairs used, varying between reactions.

**Table 2.6.3.2.1 Typical PCR Conditions**

Step	Temperature (°C)	Time (Mins)	No. Cycles
1	94	5	1
2	55 - 62	2	1
3	72	2	1
4	94	1	25 - 35
5	55 - 62	1	
6	72	2	
7	72	10	1
8	4	∞	END

### 2.6.3 DNA sequencing

All sequencing of DNA was carried out in-house using the Durham University School of Biological and Biomedical Sciences Sequencing Facility.

## 2.7 Bioinformatics

### 2.7.1 Genetic Databases

Various online genetic databases were used course of this project. The National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>) and The *Arabidopsis* Information Resource (TAIR) (<http://www.arabidopsis.org>) were used to search for and acquire gene sequences in the bioinformatic aspects of the methods.

### 2.7.2 Sequence handling software

Gene sequences used in this project were stored, handled and annotated using GENTle. This software was developed by Magnus Manske (University of Cologne, Germany) and released under the GNU General Public Licence (<http://gentle.magnusmanske.de/>).

### 2.7.3 Primer design

Novel primers for standard PCR were designed using Primer3 (Rozen & Skaletsky, 2000, pp.pp 365-386) online primer design program (<http://frodo.wi.mit.edu/primer3/>). Primers were subsequently checked for their specificity for the gene using TAIR BLAST (<http://www.arabidopsis.org/Blast/index.jsp>) and self-complementarity and potential for secondary structure formations using the Primer Stats Tool on the *Sequence Manipulation Suite* (Stothard, 2000) (<http://www.bioinformatics.org/sms2/index.html>). Primers for qRT-PCR specifically using the SYBR® Green method were designed using *Applied Biosystems* Primer Express 3.0 (*Applied Biosystems Inc.*, Foster City, USA) according to the recommendations found in the user guide. A list of primers used in this project can be found in Table 2.7.3.1.

**Table 2.7.3.1 List of primers used in this project**

Displayed are the primers used in this project and their relevant properties and intended application: either PCR or qRT-PCR.

Name	Gene	Sequence	Length (bp)	Tm (°C)	GC (%)	Product Size (bp)	Application
MYB_AT5G47390_F	AtMYB AT5G47390	GGCTGATAAAGCTTTGTGAAGGA	23	61.89	43.48	-	PCR
MYB_AT5G47390_R	"	CGACCCGATTTGCTTCTCTT	20	61.63	50	-	PCR
MYB_AT5G47390_SEQ	"	TCCTCTGTCCATGGAGTTCCTA	22	61.01	50	-	PCR
GABI-Kat Left Border (135)	"	GACAGACTGCCTAGCATTGAGT	23	65.05	47.83	-	PCR
AtSRMYB-Exon1- antisense (206)	"	GAAATCCTCAGAAGCGTAACCAT	23	63.50	43.48	-	PCR
SRMYB-Promoter- Sense (227)	"	GGATAATCACAAATGCATTAACC	24	59.68	33.33	-	PCR
MYB_T-DNA_SEQ (Rev)	"	ACTTTTCCGGATCGAACCTT	20	59.94	45	-	PCR
GUS_F	GUS	CAACGAACTGAACTGGCAGA	20	60.02	50	492	PCR
GUS_R	GUS	GCATCTTCATGACGACCAAA	20	59.65	45	492	PCR
bZIP1_F	bZIP1 AT5G49450	GGTCGCGTTTGAAGAAACAG	20	60.81	50	340	PCR
bZIP1_R	"	AAGGACGCCATTGGTTGTAG	20	59.99	50	340	PCR
GATA21_F	GATA21 AT5G56860	GAAAGCTGGGTTACCGTGAA	20	60.11	50	463	PCR
GATA21_R	"	TGGTATTCGGCAAAGAAAGG	20	60.07	45	463	PCR
CAMTA1_F	CAMTA1 AT5G09410	ATTGCATGGACCACTGTTGA	20	60.16	55	491	PCR
CAMTA1_R	"	ATCCCTGTGAGCAAGCATCT	20	59.92	55	491	PCR
PEX4_F	PEX4 AT5G2576	GCAGGCATCAAGAGCGCGACTGTTT	25	71.79	56		PCR
PEX4_R	"	GGCGAGGCGTGTATACATTTGTGCC	25	70.45	56		PCR

Name	Gene	Sequence	Length (bp)	Tm (°C)	GC (%)	Product Size (bp)	Application
LUC_F	Luc <sup>INT</sup>						PCR
LUC_R	Luc <sup>INT</sup>						PCR
bZIP1_SYBR_F	bZIP1 AT5G49450	TTCAGGTTCCGACATAGATGAGAA	24	59	42	N/A	qRT-PCR
bZIP1_SYBR_R	"	AAACGCGACCTCCTTGCA	18	59	56	N/A	qRT-PCR
GUS_SYBR_F	GUS	GAGCATCAGGGCGGCTATAC	20	58	60	N/A	qRT-PCR
GUS_SYBR_R	GUS	CGTACACTTTTCCCGGCAAT	20	59	50	N/A	qRT-PCR
PEX_SYBR_F	PEX4 AT5G2576	TCATAGCATTGATGGCTCATCCT	23	60	43	N/A	qRT-PCR
PEX4_SYBR_R	"	ACCCTCTCACATCACCAGATCTTAG	25	59	48	N/A	qRT-PCR
GATA21_F	GATA21 AT5G56860	ACCGTGAACCATTCCATACGA	21	59	48	N/A	qRT-PCR
GATA21_R	"	AAGCGTTCCCACAAGATGAGA	21	58	48	N/A	qRT-PCR
SRMYB_SYBR_F	AtMYB AT5G47390	CCGACCTACTTTTCACCATATTACC	25	58	44	N/A	qRT-PCR
SRMYB_SYBR_R	"	TCTTGGGTGGTTCAGGAACATA	22	58	45	N/A	qRT-PCR
Tubulin_F	AT5G44340	CCTGATAACTTCGTCTTTGG	20	58.93	45	~550	PCR
Tubulin_R	AT5G44340	GTGAACTCCATCTCGTCCAT	20	62.33	50	~550	PCR

## **2.7.4 Sequence alignment and searching**

The alignment of gene sequences or short sequences to each other and searching for similar or related gene was carried using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) provided by TAIR and NCBI. The alignment of multiple sequences was carried out using ClustalW2 (Larkin et al., 2007) hosted by EMBL-EBI (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). ClustalW2 was also used to produce cladograms to represent sequence similarities.

## **2.7.5 Detection of cis-acting regulatory DNA elements**

The promoter sequences (-500bp upstream of the coding region) of genes analysed in this project were acquired from TAIR using the Sequence Bulk Download and Analysis Tool (<http://www.arabidopsis.org/tools/bulk/sequences/index.jsp>) then submitted to the Database of Plant Cis-acting Regulatory DNA Elements (PLACE) (<http://www.dna.affrc.go.jp/PLACE/>) using the Signal Scan Tool (Higo et al., 1999). The data outputs were then analysed according criteria described in the results section.

## **2.8 Gene expression analysis**

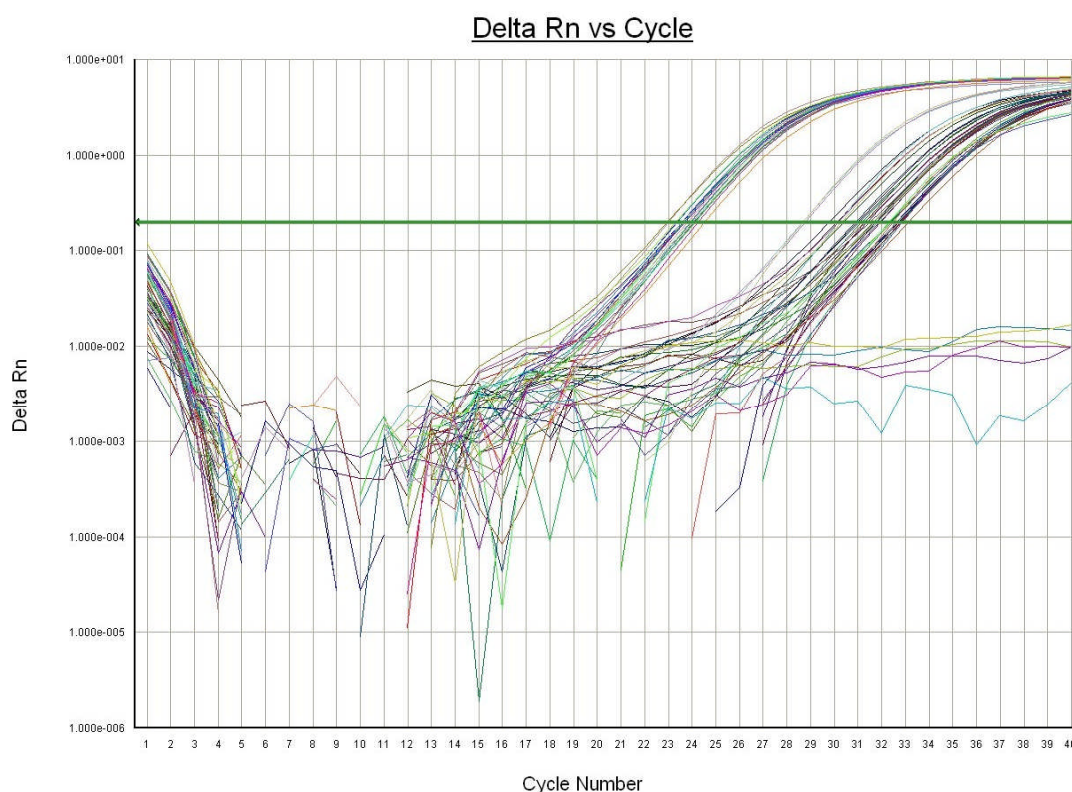
### **2.8.1 Complementary DNA (cDNA) synthesis**

The cDNA synthesised for gene expression analysis was produced as two forms: random-primed or full length synthesis. Random-primed cDNA provided templates for QRT-PCR and was synthesised using purified RNA as the template and the *Applied Biosystems* MultiScribe™ Reverse Transcriptase (Foster City, USA) primed with random hexamers according to the supplied protocol. Full length cDNA synthesis was used where comparisons of the transcript lengths were made and carried out using purified RNA as the template, synthesis using the *Epicentre* MMLV-RT 1<sup>st</sup> Strand cDNA Synthesis Kit (Madison, USA), primed using Oligo(dT)<sub>21</sub> and carried out according to the supplied protocol.

### **2.8.2 Quantitative Real-Time PCR (qRT-PCR)**

The expression of *DIN3* and the endogenous control genes were analysed by quantitative real-time PCR which directly measured the amplified PCR product to provide the relative abundance of the original cDNA transcript in each sample. The transcript abundance of the endogenous control gene was used to normalise the relative quantification to account for variation in the amount of cDNA present at the start of the reaction due to inaccuracies in the estimation of RNA concentration and differences in

reverse transcription efficiencies. In the calculation of the relative abundance of transcripts in the samples, they were compared to the transcript abundance found in the treatment control. Gene expression analysis was carried out using the *Applied Biosystems* 7300 Real-Time PCR System and the associated Sequence Detection Software (v1.4) (*Applied Biosystems Inc.*, Foster City, USA). Analysis of *DIN3* and *DIN3::LUC* expression in the linker scan lines was achieved using *Applied Biosystems* TaqMan® probes with the FAM dye. The TaqMan qRT-PCR chemistry makes use of fluorogenic-labelled probes activated by the 5' nuclease activity of Taq DNA polymerase. These oligonucleotide probes are specific to a region of the amplification products (amplicons) between the forward and reverse primers. The fluorescent reporter dye used on the 5' end of all the probes used 6-carboxyfluorescein (FAM) and minor groove binder (MGB) to increase the melting point ( $T_m$ ) and a non-fluorescent quencher (NFQ) on the 3' end. During polymerisation the probe is bound to its target sequence and whilst it remains intact the quencher dye eliminates any significant fluorescence from the reporter dye. As the polymerisation progresses the probe is cleaved by 5' nuclease activity of Taq DNA polymerase. Probe cleavage separates the reporter dye from the quencher rendering the fluorescent reporter signal detectable. Polymerisation of the amplicon continues unhindered. As other reporter dye molecules are cleaved from their probes, the intensity of the detected fluorescent signal increases proportional to the amount of amplicon produced (*Applied Biosystems*, n.d.). All other gene expression used SRBYB® Green probes and associated method, whereby the SYBR Green I dye binds to each new copy of double-stranded DNA and so an increase in fluorescence intensity is proportionate to the amount of amplicon produced (*Applied Biosystems*, n.d.). The sequence detection software provided 'Threshold cycle' or  $C_T$  values (fig. 2.8.2.1) which were the fractional number of reaction cycles required to reach a pre-defined fluorescence threshold. These values were processed manually to give a 'relative quantitation' of gene expression or RQ value (*Applied Biosystems*, 2007). The  $C_T$  method requires validation demonstrating that the efficiencies of the target and endogenous control amplifications are approximately equal (*Applied Biosystems*, n.d.; Livak & Schmittgen, 2001). Statistics were performed on the gene expression data according to the *Applied Biosystems* User Bulletin 'Relative Quantitation (RQ) Algorithms in Applied Biosystems Real-Time PCR Systems Software' (PN 4378622). All qRT-PCRs were carried out in 96-well semi-skirted, qPCR compatible plates (*STARLAB (UK) Ltd.*, Milton Keynes, UK).



**Figure 2.8.2.1 Example amplification plot for qRT-PCR gene expression analysis**

The SYBR Green® or Taqman® methods provide fluorescence which is measured by the Applied Biosystems RT-PCR Analysis System at the end of every amplification cycle. The threshold is set to where the plot for each reaction and probe is increasing exponentially. This gives the 'threshold cycle' or  $C_T$  value for gene expression analysis.

### **2.8.2.1 TaqMan® Probes**

The use of the TaqMan® probes was restricted to cDNA samples from experiments using linker scan line plants and in the confirmation of *MYB* overexpression. Probes used against these samples targeted *DIN3* (AT3G06850), *PEX4* (AT5G25760) and the reporter gene *DIN3::LUC*. Probes to *DIN3* (Part No. 4351372) and *DIN3::LUC* (Part No. 4331348 - designed using *Applied Biosystems File Builder Software*) were produced to order by *Applied Biosystems* (Foster City, USA), whilst *PEX4* (Part No. 4351372) was an inventoried probe. All TaqMan® probes contained the FAM and NFQ dyes. qRT-PCRs carried out using these probes were set up with the TaqMan® Gene Expression Master Mix (*Applied Biosystems Inc.*, Foster City, USA) and a 1-in-10 dilution (optimised after validation of amplification efficiencies) of cDNA synthesised using MultiScribe™ Reverse Transcriptase (*Applied Biosystems Inc.*, Foster City, USA) according to the manufacturer protocol.

### **2.8.2.2 SYBR® Green method**

The SYBR® Green qRT-PCR method relies on amplification of a PCR product using gene specific primers, which were designed using *Applied Biosystems Primer Express*

3.0 then synthesised by *Invitrogen Ltd.* (Paisley, UK). SYBR® Green qRT-PCRs were carried out using the *Bioline SensiMix SYBR Kit* (*Bioline UK Ltd.*, London, UK). Unlike the TaqMan® method, SYBR® Green primers require optimisation to determine their optimum concentration in the reaction mix and the corresponding optimum concentration of cDNA template. Once the optimum concentrations had been determined the QRT-PCRs were set up using the *Bioline SensiMix SYBR Kit* according to the supplied protocol.

## **2.9 Sugar assays**

Assaying sugar content in plant tissues was carried out using the Glucose Assay Kit and Sucrose Assay Kit (*Sigma-Aldrich Company Ltd.*, Dorset, UK) for glucose and sucrose assays respectively. Both kits used a hexokinase-based assay. All sugar values obtained from the assays were standardised against the protein content of the samples the readings were processed using the formula and method described in the protocol provided with the kit to give the concentration of sucrose in the tissue samples as mg/ml. These data were standardised against their protein content (2.10 Protein assays).

### **2.9.1 Extraction and analysis**

#### **2.9.1.1 Glucose**

The protocol provided with the Glucose Assay Kit did not have a prescribed method for the extraction of glucose from plant tissues. A modification of the extraction protocol was produced by Kerry Franklin (personal communication). 250mg of plant tissue was used in the glucose assays. A mortar of appropriate size was pre-chilled with liquid nitrogen and 250mg of plant tissue transferred from the liquid nitrogen (either freshly harvested or stored at -80°C) to the mortar then ground into a fine powder. Next 1ml of MQ water was added to the mortar and washed around its sides. The water froze rapidly and took about 10 – 15 minutes to thaw during which time further samples can be processed. Once completely thawed the lysate was transferred to a 15-ml centrifuge tube and incubated for 30 minutes in a 60°C water bath after which all samples were centrifuged at maximum speed for 15 minutes. The supernatant was then used in the assay according to the protocol provided with the kit. The absorbance of the samples were measured at 340nm using a *BOECO S-20* spectrophotometer (*Boeckel + Co (GmbH + Co)*, Hamburg, Germany). The readings were processed using the formula and method described in the protocol provided with the kit to give the concentration of glucose in the tissue samples as mg/ml. These data were standardised against their

protein content the readings were processed using the formula and method described in the protocol provided with the kit to give the concentration of sucrose in the tissue samples as mg/ml. These data were standardised against their protein content (2.10 Protein assays).

### **2.9.1.2 Sucrose**

Sucrose assays were carried out using the extraction method described above, then according to the supplied protocol. As with the glucose assays, the absorbance of the samples was measured at 340nm and the readings were processed using the formula and method described in the protocol provided with the kit to give the concentration of sucrose in the tissue samples as mg/ml. These data were standardised against their protein content (2.10 Protein assays).

## **2.10 Protein assays**

Protein assays were carried out using the *Bio-Rad* DC Protein Assay Kit (*Bio-Rad Laboratories Ltd.*, Hemel Hempstead, UK).

### **2.10.1 Extraction and analysis**

The protein assays were carried out according to the protocol supplied with the *Bio-Rad* DC Protein Assay Kit. Protein assays carried out on plant tissue samples extracted using a detergent based cell lysis buffer, such as used in luminometry assays (2.11.1 Tissue preparation), then the sample was diluted 1-in-2 with MQ water. Samples extracted without a detergent were used directly in the assay without dilution. The absorbance of the samples were measured at 750nm and the readings processed by using the equation of the line of the graph of standards known protein concentration (bovine serum albumin).

## **2.11 Luciferase reporter gene**

Plant tissue samples expressing the *DIN3::LUC* gene were also analysed by luminometry, that is: measuring the amount of luminescence produced with the addition of luciferin to a lysate of 10, 7-day-old seedlings grown under long-day condition having been treated with 6 hour of light or darkness at 20°C. This measurement provided an assessment of the *DIN3::LUC* gene expression following the treatment. Seedlings expressing an unmodified version of the *DIN3::LUC* gene were grown and treated alongside those of the linker scan lines and included in the assay to deduct their luminescence readings from those of the linker scan lines.

### **2.11.1 Tissue preparation and luminescence assays**

Two samples of 10, 7-day-old seedlings of each transformant line, of each linker scan line and of each treatment (six hours of light or darkness at 20°C) were harvested following the end of the treatment to a 1.5-ml microcentrifuge tube and then frozen in liquid nitrogen. The samples were then stored at -80°C until an appropriate time to carry out the luminescence assay. The frozen seedlings were ground with a pellet pestle (*Anachem Ltd.*, Luton, UK) for a few seconds and then 200µl of *Promega* 1x Luciferase Cell Culture Lysis Reagent (*Promega UK Ltd.*, Southampton, UK) added. The pestle was then attached to a cordless pestle motor (*Anachem Ltd.*, Luton, UK) and the seedlings homogenised to a lysate. The samples in the microcentrifuge tubes were then centrifuged at maximum speed for 2 minutes. The supernatant was used in the luminescence assays. The luminescence assays were carried out using a photo-multiplier tube based luminometer of custom design (Knight & Knight, 1995). 80µl of each sample was transferred to 1ml sample cuvette to which was added 20µl of *Promega* Luciferase Assay Reagent (*Promega UK Ltd.*, Southampton, UK) and mixed by pipette. The sample was then assayed using the luminometer for 20 seconds and the luminescence reading recorded.

### **2.11 Statistics**

Descriptive statistics were carried out using the appropriate functions available in Microsoft Office Excel® 2003/7. Student's *t*-tests were performed using the data analysis toolkit addin for Microsoft Office Excel®. Analyses of variance (ANOVA) were carried out using SPSS Statistics for Windows 15 (*SPSS Inc.*, Chicago, USA).

### 3.1 Analysis of *DIN* genes promoter motifs

The corpus of published research regarding cis-acting regulatory elements found in promoter regions is always increasing. Several databases have been established which enable the input of promoters sequence to be searched for motifs of previously described cis-acting regulatory elements. This can be a powerful tool for preliminary *in silico* analysis of the factors which may be involved in the regulation of the gene of interest. The aim of the promoter motif analysis was to investigate whether the *DIN* genes have common regulatory motifs that help explain their shared response to light and whether they exhibit other collective regulatory elements. The identification of these regulatory motifs provides candidate transcription factors which then may be targeted more directly in latter experiments to better determine the nature and extent of the regulatory role indicated by the promoter analysis. Sections of the (500bp upstream of the ATG) promoter regions of the *DIN* genes, were acquired from The *Arabidopsis* Information Resource (TAIR) website (<http://www.arabidopsis.org>) using a tool to download sequences selectively from their database. The sequences of the 500bp portions of *DIN* genes: 1 – 4, 6 and 9 – 11 (i.e. all the *DIN* genes that were available) were downloaded and then submitted to Plant Cis-acting Regulatory DNA Elements (PLACE) online database signal scanner (<http://www.dna.affrc.go.jp/PLACE>) (Higo et al., 1999). The results of this analysis was then compared using the TAIR Statistical Motif Analysis (<http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp>) with 20 randomly selected *A. thaliana* genes (using RSAT (van Helden, 2003; Thomas-Chollier et al., 2008)) to determine whether the frequency of regulatory motifs in the *DIN* genes were usual amongst others in the genome and so better assess the extent to which the motifs might contribute towards the regulation of the *DIN* genes. Finally focussing on the subject of this study, *DIN3*, the motifs present in the -300 to -230bp portion of the *DIN3* promoter were analysed using PLACE to indentify the loci of known cis-acting regulatory elements, which may be involved in the response of *DIN3* expression to darkness, to aid the interpretation of the results from unbiased mutations of that section of the promoter.

**Table 3.1.1 Frequency of motifs with relevance to light/dark or cold regulation found in the promoters of the *DIN* genes.**

The data presented in this table were assembled from -500bp promoter sequences of *DIN* genes 1 – 4 (AT4G35770, AT3G60140, AT3G06850, AT3G13450), 6 (AT3G47340) and 9 – 11 (AT1G67070, AT5G20250, AT3G49620) (obtained from The Arabidopsis Information Resource (TAIR) website), which were subsequently submitted to Plant Cis-acting Regulatory DNA Elements (PLACE) (<http://www.dna.affrc.go.jp/PLACE>) online database signal scanner (Higo et al., 1999). The motifs associated with light/dark or cold regulation occurring in the promoters of *DIN* genes are presented in this table with the frequency of the specific signal sequence of a recognised consensus or at least possessing a core consensus of a described cis-acting regulatory element. The promoter of the genes in which they are found are indicated and brief notes from the literature added for explanation as to their function or association. Probabilities indicate the likelihood that the presence of the motif is due to chance; determined using the TAIR Statistics Motif Analysis Tool (<http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp>).

In addition to the usual nucleotide base letter symbols, the following letters represent loci where one base may be substituted for one or more others in the consensus of the motif: B = C, G or T; D = A, G or T; H = A, C or T; K = G or T; M = A or C; N = A, C, G or T; R = A or G; S = C or G; V = A, C or G; W = A or T; Y = C or T. (R-C) stands for reverse complemented; (P) for partial coverage but include the core consensus.

Motif Name	Consensus	Signal Sequence	Presence in <i>DIN</i> genes	Frequency in <i>DIN</i> genes	Probability	Notes from Literature
ABRERATCAL	MACGYGB ACGT	(R-C) (P) CACGT	DIN2, 9	2	0.104	ABRERATCAL, a Ca <sup>2+</sup> -responsive cis-element found in the upstream regions of 162 Ca <sup>2+</sup> -responsive upregulated genes (Kaplan et al., 2006) from (Gupta et al., 2008) K <sup>+</sup> Transporters.
		(R-C) ACGCGTG	DIN3	1		
		(P) ACGTGG	DIN4, 10	2	1.06 x 10 <sup>-4</sup>	
		CACGTGT	DIN6	1	2.62 x 10 <sup>-2</sup>	
		CACGTGG	DIN6	1	1.06 x 10 <sup>-4</sup>	
		(R-C) ACAGTG	DIN6, 11	3		
(P) ACGTGT	DIN10	1	2.62 x 10 <sup>-2</sup>			
CCAATBOX1	CCAAT	(R-C) ATTGG	DIN1, 6	4	0.958	CCAATBOX1 motif found in ~25% eukaryotic promoters; CCAATBOX1 motif bound by Heme Activator Protein (HAP) complex. Isoforms of HAP complex interact with CONSTANS (CO) which is involved in regulating flowering time (Wenkel et al., 2006)
		CCAAT	DIN3, 4, 6, 10, 11	8	0.152	

Motif Name	Consensus	Signal Sequence	Presence in <i>DIN</i> genes	Frequency in <i>DIN</i> genes	Probability	Notes from Literature
CGCGBOXAT	<u>VCGCGB</u>	<u>ACGCGT</u>	DIN3	2 (Palindromes)		The CG-1 domain recognizes the core consensus sequence, <u>vCGCGB</u> , referred to as the CG-1 element (Silva, 1994). In Roles for Arabidopsis CAMTA Transcription Factors in Cold-Regulated Gene Expression and Freezing Tolerance (Doherty et al., 2009).
DPBFCOREDCCDC	ACAC <u>NNG</u>	ACAC <u>ATG</u> CGCGTGT ACAC <u>GTG</u>	DIN1 DIN3, 6 DIN6, 11	1 2 3	0.199 $1.24 \times 10^{-2}$ $2.62 \times 10^{-2}$	DPBFCORE (ACACNNG) is a core-binding motif of a novel class of bZIP transcription factors (DPBF-1 and DPBF-2), found in the carrot Dc3 gene promoter; ABA inducible (Kim et al., 1997).
GATABOX	GATA	GATA (R-C) TATC	DIN1, 2, 3, 4, 6, 9, 11 DIN1, 2, 3, 4, 6, 9, 10, 11	25 31	1 1	GATA motif is strongly implicated in light regulated genes; highly conserved in genes such as chlorophyll a/b binding protein and the small subunit of rubisco (Gilmartin et al., 1990).
GT-1 BOX	<u>GRWAAW</u>	(R-C) TTTACC (R-C) ATTATC (R-C) TTTATC GATAAT GAAAAA (R-C) ATTTCC (R-C) ATTACC GGAAAA (R-C) TTTTTC GATAAA (R-C) ATTTTC (R-C) TTTTCC GAAAAT GGTAAT	DIN1 DIN1, 11 DIN1, 4, 10, 11 DIN1, 2, 3, 11 DIN2, 3, 6, 10 DIN2, 11 DIN3, 6 DIN3, 4, 6, 11 DIN4, 6, 11 DIN4, 6 DIN6, 11 DIN10 DIN11	1 2 7 4 2 2 1 2 7 4 2 2 1 1	- 0.199 0.168 0.199 0.244 0.258 - 0.263 0.244 0.168 0.11 0.263 0.11 -	GT-1 Motif over-represented in the promoter of the light-regulated gene for the small subunit of rubisco (Gilmartin et al., 1990; Le Gourrierec et al., 1999; Lam & Chua, 1990).
IBOX	GATAAG	GATAAG (R-C) CTTATC	DIN1, 3, 4, 6, 9, 11 DIN1, 6, 9, 11	6 7	$2.64 \times 10^{-3}$ $2.64 \times 10^{-3}$	I Boxes have a GATA motif as their core and are similarly over-represented in the promoters of light regulated genes such as the small subunit of rubisco (Gilmartin et al., 1990; Giuliano et al., 1988).

Motif Name	Consensus	Signal Sequence	Presence in <i>DIN</i> genes	Frequency in <i>DIN</i> genes	Probability	Notes from Literature
MYB2CONSENSUS	<u>YAACKG</u>	(R-C) CCGTTG (R-C) CAGTTA <u>CAACGG</u> CAACTG	DIN3 DIN9 DIN10 DIN10	1 1 1 1	- - - -	The <i>AtMYB2</i> recognition site is found in the promoters of drought responsive genes. The <i>AtMYB2</i> gene is itself upregulated by drought and ABA treatments (Abe et al., 2003).
MYCCONSENSUSAT	CANNTG Forms palindromes on either strand	CATATG CAAATG CAAGTG CAATTG	DIN1, 6 DIN2 DIN9 DIN3, 10, 11	10 1 3 6	- $7.32 \times 10^{-2}$ 0.125 -	MYC motif binding site for <i>Inducer of CBF Expression (ICE)</i> . <u>C</u> - repeat <u>B</u> inding <u>F</u> actor family of transcription factors mediate cold acclimation (Chinnusamy et al., 2003).
MYBST1	GGATA	GGATA  (R-C) TATCC	DIN1, 2, 3, 6  DIN1, 6, 10, 11	4  10	$2.09 \times 10^{-2}$  $1.49 \times 10^{-2}$	Binding site of DNA binding protein MybSt1 – a transcriptional activator (Baranowskij et al., 1994). TATCC part of the TATCCA element of the Sugar Responsive Sequence (SRS) found in promoter of rice $\alpha$ -Amylase gene (Lu et al., 1998). TATCCA element bound by three rice sugar responsive Myb transcriptional activator proteins: OsMYBS1, OsMYBS2 & OsMYBS3 (Lu et al., 2002).
LTRE1HVBLT49/ LTRECOREATCOR15 (CRT/DRE)	CCGANN	CCGAAA CCGAC (R-C) GTCGG	DIN1 DIN1, 4 DIN3	1 2 1	$9.1 \times 10^{-2}$ - -	<u>Low Temperature Responsive Element (LTRE)</u> is found in low temperature responsive gene ( <i>blt.49</i> ) in <i>Hordeum Vulgare</i> (Dunn et al., 1998).
SORLIP1	<u>GSSMC</u>	<u>GCCAC</u> (R-C) GTGGC <u>GGGCC</u> (R-C) GGCCC	DIN1 DIN3, 4, 10 DIN1 DIN1	1 3 2 1	$3.56 \times 10^{-2}$ $3.56 \times 10^{-2}$ - -	<u>Sequences over-represented in light-induced promoters 1</u> (SORLIP1) is a phyA motif which is over-represented in light-induced genes (Hudson & Quail, 2003; Jiao et al., 2005).

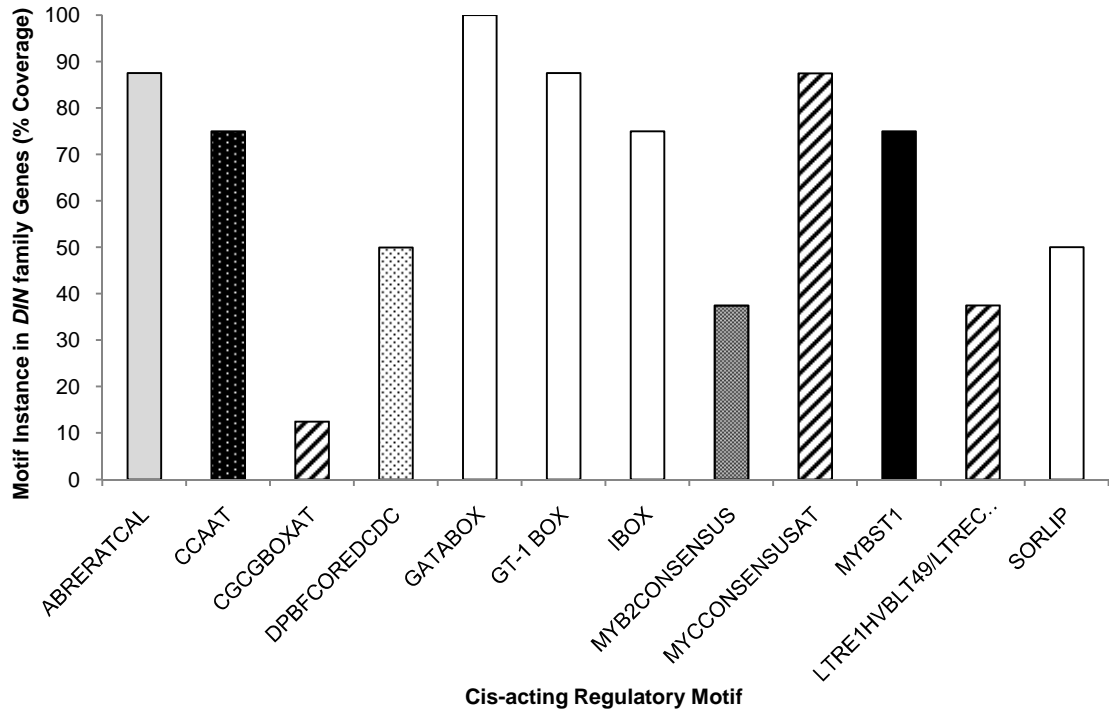
**Table 3.1.2 Frequency of *DIN* motifs in 20 random *A. thaliana* genes**

The data presented in this table were assembled from -500bp promoter sequences of 20 random *A. thaliana* genes (AT5G56570, AT5G45510, AT3G28690, AT2G42210, AT3G58790, AT1G31340, AT1G68430, AT4G35950, AT5G13300, AT3G51830, AT5G36680, AT5G05160, AT1G20620, AT5G10980, AT3G10330, AT3G63370, AT4G05360, AT5G11230, AT2G43945, AT4G14690) chosen using RSAT (van Helden, 2003; Thomas-Chollier et al., 2008) then the -500bp promoter regions analysed using TAIR Statistics Motif Analysis Tool (<http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp>) to determine the frequency of each motif amongst the 20 genes. The same motifs as displayed in Table 3.1.1 were searched for amongst the 20 genes to determine their frequency in promoters of a random cross-section of *A. thaliana* genes.

Motif Name	Consensus	Signal Sequence	Frequency in 20 random genes	Notes from Literature
ABRERATCAL	<u>M</u> AC <u>G</u> Y <u>G</u> B ACGT	(R-C) (P) CACGT (R-C) ACGCGTG (P) ACGT <u>G</u> G <u>C</u> ACGT <u>G</u> T <u>C</u> ACGT <u>G</u> G (R-C) ACAGTG (P) ACGT <u>G</u> T	12 0 3 13 9 0 7	ABRERATCAL, a Ca <sup>2+</sup> -responsive cis-element found in the upstream regions of 162 Ca <sup>2+</sup> -responsive upregulated genes (Kaplan et al., 2006) from (Gupta et al., 2008) K+ Transporters.
CCAATBOX1	CCAAT	(R-C) ATTGG CCAAT	21 21	CCAATBOX1 motif found in ~25% eukaryotic promoters; CCAATBOX1 motif bound by Heme Activator Protein (HAP) complex. Isoforms of HAP complex interact with CONSTANS (CO) which is involved in regulating flowering time (Wenkel et al., 2006)
CGCGBOXAT	<u>V</u> CG <u>C</u> G <u>B</u>	<u>A</u> CG <u>C</u> G <u>T</u>	0	The CG-1 domain recognizes the core consensus sequence, vCGCGb, referred to as the CG-1 element (Silva, 1994). In Roles for Arabidopsis CAMTA Transcription Factors in Cold-Regulated Gene Expression and Freezing Tolerance (Doherty et al., 2009).

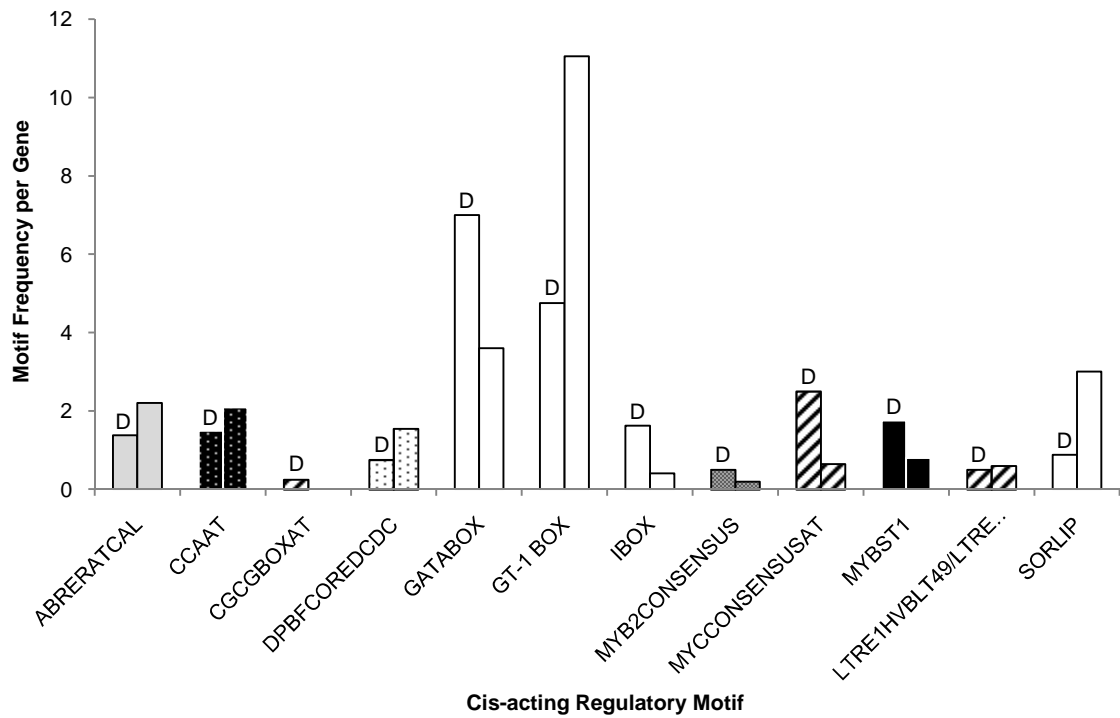
Motif Name	Consensus	Signal Sequence	Frequency in 20 random genes	Notes from Literature
DPBFCOREDCDC	ACAC <u>N</u> NG	ACACATG CGCGTGT ACAC <u>G</u> TG	15 3 13	DPBFCORE (ACACNNG) is a core-binding motif of a novel class of bZIP transcription factors (DPBF-1 and DPBF-2), found in the carrot Dc3 gene promoter; ABA inducible (Kim et al., 1997).
GATABOX	GATA	GATA (R-C) TATC	36 36	GATA motif is strongly implicated in light regulated genes; highly conserved in genes such as chlorophyll a/b binding protein and the small subunit of rubisco (Gilmartin et al., 1990).
GT-1 BOX	<u>GR</u> WAAW	(R-C) TTTACC (R-C) ATTATC (R-C) TTTATC GATAAT GAAAAA (R-C) ATTTCC (R-C) ATTACC GGAAAA (R-C) TTTTTC GATAAA (R-C) ATTTTC (R-C) TTTTCC GAAAAT GGTAAT	8 12 11 12 42 3 4 17 42 11 19 17 19 4	GT-1 Motif over-represented in the promoter of the light-regulated gene for the small subunit of rubisco (Gilmartin et al., 1990; Le Gourrierec et al., 1999; Lam & Chua, 1990).
IBOX	GATAAG	GATAAG (R-C) CTTATC	4 4	I Boxes have a GATA motif as their core and are similarly over-represented in the promoters of light regulated genes such as the small subunit of rubisco (Gilmartin et al., 1990; Giuliano et al., 1988).
MYB2CONSENSUS	<u>Y</u> AACK <u>G</u>	(R-C) CCGTTG (R-C) CAGTTA <u>CAAC</u> G  <u>CAAC</u> TG	0 4 0  0	The <i>AtMYB2</i> recognition site is found in the promoters of drought responsive genes. The <i>AtMYB2</i> gene is itself upregulated by drought and ABA treatments (Abe et al., 2003).

Motif Name	Consensus	Signal Sequence	Frequency in 20 random genes	Notes from Literature
MYCCONSENSUSAT	CANNTG Forms palindromes on either strand	CATATG CAAATG CAAGTG CAATTG	0 7 0 6	MYC motif binding site for <i>Inducer of CBF Expression (ICE)</i> . <i>C - repeat Binding Factor</i> family of transcription factors mediate cold acclimation (Chinnusamy et al., 2003).
MYBST1	GGATA	GGATA  (R-C) TATCC	8  8	Binding site of DNA binding protein MybSt1 – a transcriptional activator (Baranowskij et al., 1994). TATCC part of the TATCCA element of the Sugar Responsive Sequence (SRS) found in promoter of rice $\alpha$ -Amylase gene (Lu et al., 1998). TATCCA element bound by three rice sugar responsive Myb transcriptional activator proteins: OsMYBS1, OsMYBS2 & OsMYBS3 (Lu et al., 2002).
LTRE1HVBLT49/ LTRECOREATCOR15 (CRT/DRE)	CCGANN	CCGAAA CCGAC (R-C) GTCGG	0 6 6	<u>L</u> ow <u>T</u> emperature <u>R</u> esponsive <u>E</u> lement (LTRE) is found in low temperature responsive gene ( <i>blt.49</i> ) in <i>Hordeum Vulgare</i> (Dunn et al., 1998).
SORLIP1	GSSMC	GCCAC (R-C) GTGGC GGGCC (R-C) GGCCC	15 15 15 15	<u>S</u> equences <u>o</u> ver- <u>r</u> epresented in <u>l</u> ight- <u>i</u> nduced <u>p</u> romoters <u>1</u> (SORLIP1) is a phyA motif which is over-represented in light-induced genes (Hudson & Quail, 2003; Jiao et al., 2005).



**Figure 3.1.1 Occurrence of cis-acting regulatory motifs in the *DIN* genes**

The bars represent the proportion (expressed as a percentage) of the -500bp promoter regions of the *DIN* genes (Table 3.1.3), out of a total of eight surveyed, in which the various cis-acting regulatory motifs, implicated in light/dark (white bars) or cold regulation (striped), in addition to  $Ca^{2+}$  responsive (grey), flowering time (black with dots), ABA/bZIP inducible (white with dots), drought responsive (checked) and MybSt1 sites (black), occur. These data were produced from outputs of analyses carried out using PLACE (Higo et al., 1999). Median number of *DIN* genes for motif to be found in = 6.



**Figure 3.1.2 Per-gene frequency of cis-acting regulatory motifs in *DIN* and 20 random genes**

The grey bars represent the number of instances (frequency) in which each cis-acting regulatory motif, implicated in light/dark (white bars) or cold regulation (striped), in addition to  $Ca^{2+}$  responsive (grey), flowering time (black with dots), ABA inducible (white with dots), drought responsive (checked), and MybSt1 sites (black), were found in the -500bp promoter regions of *DIN* genes (D) and 20 randomly selected *A. thaliana* genes (Table 3.1.3). These data were produced from outputs of analyses carried out using PLACE (Higo et al., 1999).

**Table 3.1.3 Motif frequencies in -500bp promoter regions of *DIN* and 20 random *A. thaliana* genes**

The data presented in this table used the frequencies of cis-acting regulatory motifs identified in the <i>DIN</i> (Table 3.1.1) and 20 randomly selected <i>A. thaliana</i> genes (Table 3.1.2) to generate the median, total and total per gene motif frequencies group according to function or regulatory association (motif type).						
Motif type	8 <i>DIN</i> genes			20 random <i>A. thaliana</i> genes		
	Median	Total	Per gene total	Median	Total	Per gene total
Light	25.5	114	14.25	66	361	18.05
Cold	4	26	3.25	12	25	1.25
Flowering	12	42	5.25	42	42	2.1
ABA/bZIP	6	6	0.75	31	31	1.55
Drought	4	4	0.5	4	4	0.2
MybSt1	14	14	1.75	16	16	0.8
Calcium	11	11	1.375	44	44	2.2

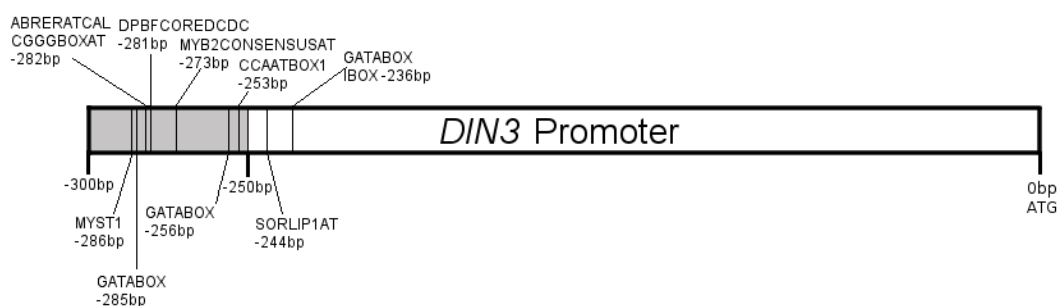
**Table 3.1.4 Motifs found in the -300 to -230bp of the *DIN3* promoter with relevance to light/dark or cold regulation**

The data presented in this table were assembled from the *DIN3* promoter sequence (obtained from The Arabidopsis Information Resource (TAIR) website), which were subsequently submitted to Plant Cis-acting Regulatory DNA Elements (PLACE) (<http://www.dna.affrc.go.jp/PLACE>) online database signal scanner (Higo et al., 1999). The motifs occurring in the -300 to -230bp region (fig. 3.1.3) of the *DIN3* promoter, relevant to light/dark or cold regulation, are presented in this table with their locus and brief notes from the literature added for explanation as to their function or association. (R-C) stands for reverse complemented.

Motif Name	Consensus	Sequence	Locus	Function
ABRERATCAL	<u>M</u> ACGY <u>B</u>	(R-C) ACGCGTG	-282bp	ABRERATCAL, a Ca <sup>2+</sup> -responsive cis-element found in the upstream regions of 162 Ca <sup>2+</sup> -responsive upregulated genes (Kaplan et al., 2006) from (Gupta et al., 2008) K+ Transporters.
CAATBOX1	CAAT	CAAT	-253bp	CCAATBOX1 motif found in ~25% eukaryotic promoters; CCAATBOX1 motif bound by <u>H</u> eme <u>A</u> ctivator <u>P</u> rotein (HAP) complex. Isoforms of HAP complex interact with <u>C</u> ONSTANS (CO) which is involved in regulating flowering time (Wenkel et al., 2006).
CGCGBOXAT	<u>V</u> CGC <u>B</u>	<u>A</u> CGC <u>I</u>	-282bp	The CG-1 domain recognizes the core consensus sequence, vCGCGb, referred to as the CG-1 element (Silva, 1994). In Roles for Arabidopsis CAMTA Transcription Factors in Cold-Regulated Gene Expression and Freezing Tolerance (Doherty et al., 2009).
DPBFCOREDCDC	ACAC <u>N</u> NG	(R-C) CGCGTGT	-281bp	DPBFCORE (ACACNNG) is a core-binding motif of a novel class of bZIP transcription factors (DPBF-1 and DPBF-2), found in the carrot Dc3 gene promoter; ABA inducible (Kim et al., 1997).
GATABOX	GATA	GATA GATA GATA	-285bp -256bp -236bp	GATA motif is strongly implicated in light regulated genes; highly conserved in genes such as chlorophyll a/b binding protein and the small subunit of rubisco (Gilmartin et al., 1990).
IBOX	GATAAG	GATAAG	-236bp	I Boxes have a GATA motif as their core and are similarly over-represented in the promoters of light regulated genes such as the small subunit of rubisco (Gilmartin et al., 1990; Giuliano et al., 1988).
MYB2CONSENSUSAT	<u>Y</u> AACK <u>G</u>	(R-C) <u>C</u> CGT <u>I</u> G	-273bp	The AtMYB2 recognition site is found in the promoters of drought responsive genes. The AtMYB2 gene is itself upregulated by drought and ABA treatments (Abe et al., 2003).
MYBST1	GGATA	GGATA	-286bp	Binding site of DNA binding protein MybSt1 – a transcriptional activator (Baranowskij et al., 1994). TATCC part of the TATCCA element of the Sugar Responsive Sequence (SRS) found in promoter of rice α-Amylase gene (Lu et al., 1998). TATCCA element bound by three rice sugar responsive Myb transcriptional activator proteins: OsMYBS1, OsMYBS2 & OsMYBS3 (Lu et al., 2002).
SORLIP1	GCCAC	(R-C) GTGGC	-244bp	SORLIP1 is a phyA motif which is over-represented in light-induced genes (Hudson & Quail, 2003; Jiao et al., 2005).

**Table 3.1.5 Cis-acting regulatory motifs present at the loci of the linker scan lines**

Linker Scan Line	Locus	Motif
1	-297bp	-
2	-293bp	-
3	-289bp	MYBST1
4	-285bp	MYBST1, GATA, DPBFCOREDCDC (-ve strand)
5	-281bp	ABRERATCAL, CGGGBOXAT, DPBFCOREDCDC
6	-277bp	ABRERATCAL, CGGGBOXAT
7	-273bp	MYB2CONSENSUSAT
8	-269bp	MYB2CONSENSUSAT
9	-265bp	-
10	-261bp	-
11	-257bp	GATABOX
12	-253bp	CCAATBOX1



**Figure 3.1.3 Map of *DIN3* promoter region annotated with loci of cis-acting regulatory motifs**

The loci (first base) of the cis-acting regulatory motifs found in the -300 to -230bp portion of the *DIN3* promoter region described in Tables 3.1.4 and 3.1.5 are annotated on this map (thin black lines). The portion of the promoter region coloured in grey indicates that covered by the linker scan analyses, the loci of which and affected motifs are described in Table 3.1.5. Motif DPBFCOREDCDC is found on the antisense strand and hence the locus indicated is its final base.

### ***Review of the analytical approach to the promoter motif analysis***

The scope of this project limited the promoter analysis to the search for motifs relevant to light/dark or cold regulation. The output from PLACE provides a brief description of the putative association of the cis-acting regulatory elements with transcription factors along with supporting references. Those motifs included in the promoter analysis were selected primarily due to their over-representation and subordinately because they were described as being cis-acting regulatory elements which were indicated either to have associations with transcription factors known to be involved in light/dark or cold

regulation or were themselves over-represented in the promoters of genes which are light/dark or cold regulated. Motifs not meeting these criteria were ignored. Firstly the members of the *DIN* genes, whose -500bp portions of their promoters were submitted to PLACE and analysed for the occurrence, frequency and the exact signal sequence of relevant cis-acting regulatory elements (Table 3.1.3; figs. 3.1.1). The frequencies of the motifs identified in the first analysis were compared with their frequencies in 20 randomly selected *A. thaliana* genes (Tables 3.1.2/3/4; fig. 3.1.2). The -300 to -230bp (fig. 3.1.3) of the *DIN3* promoter was submitted to PLACE for the same analysis, the exact loci of the cis-acting elements within this portion, 5' to 3', were also recorded (Table 3.1.4). Finally the loci of the motifs identified in the -300 to -230bp of the *DIN3* promoter were matched the loci of the 12 linker scan lines (Table 3.1.5).

### ***Analysis of the -500bp section of the promoters of the DIN genes***

Charting the occurrence of the cis-acting regulatory motifs (fig. 3.1.1), indicated by the PLACE analysis to be involved in either light/dark or cold gene regulation, revealed a variation between them with respect to how many of which were found in the eight members of the *DIN* genes (Table 3.1.1). The motifs may also be grouped according to their regulatory function as described in the literature (Table 3.1.1; fig. 3.1.1). Ranked according to the median occurrence of the regulatory group in the *DIN* genes, Ca<sup>2+</sup> responsive motif (ABRERATCAL) with 7/8 genes; the light associated motifs, the largest group with four members: GATABOX, GT-1 BOX, IBOX and SORLIP1 had the largest median occurrence found in 6.5 out of the eight *DIN* genes (Table 3.1.3; fig. 3.1.1). This was followed by flowering (CCAATBOX1) and the MYBST1 binding site, both found in 6/8 genes; ABA/bZIP induction (DPBF COREDCDC) with 4/8 then the cold (CGCGBOXAT, MYCCONSENSUSAT and the LTRE-type motifs) and drought responsive motifs (MYB2CONSENSUS) with 3/8 (Table 3.1.3; fig. 3.1.1). It is worth noting that *DIN3* is the only member of the *DIN* genes which possesses all three of the cold regulatory motifs analysed and all four of the light regulatory motifs were found in *DIN3* (Table 3.1.3; fig. 3.1.1).

Charting the incidence (frequency) at which each of the 12 motifs occur in the *DIN* genes (fig. 3.1.2) produces a very similar pattern to that seen when the proportion of the *DIN* genes that each motif was found in was analysed (fig. 3.1.1). This suggests that the abundance of the motifs in the *DIN* genes is at least partly due to the proportion of the eight *DIN* genes in which they are found. When ranked in order of frequency, the groups with the greatest incidence were the light regulatory motifs, flowering, cold

responsive, the MybSt1 binding motif, the Ca<sup>2+</sup> responsive motif, ABA/bZIP inducible motif and least common the drought responsive motif (Table 3.1.3).

### **Comparison with 20 random *A. thaliana* genes**

Considering that the high incidence of light regulatory motifs appears to be similar between the *DIN* and group of 20 random genes suggests that the dominance light regulatory motifs is not necessarily associated with *DIN* genes per se, but perhaps a commonly shared feature in the regulation of plant genes (Table 3.1.3; fig. 3.1.2). The incidence of cold, flowering, drought and the MybSt1 binding sites were all 2.2 times greater amongst the *DIN* genes than the group of 20 random genes (Table 3.1.3; fig. 3.1.2). This difference is potentially more significant, suggesting that these regulatory motifs are associated with the *DIN* genes more strongly than others in the *A. thaliana* genome, thereby alluding to a deeper regulatory role for their associated transcription factors. The incidence ABA/bZIP associated motif and calcium responsive element were depreciated amongst the *DIN* genes, with these elements 2.1 and 1.6 times more common amongst the 20 random genes respectively (Table 3.1.3; fig. 3.1.2).

### **Analysis of the -300 to -230bp portion of the *DIN3* promoter**

As with the results of the cis-acting regulatory element analysis of the -500bp promoters of the eight members of the *DIN* genes, the results for the -300 to -230bp of the *DIN3* promoter can be divided along the types of regulation. The largest group is made up of three motifs GATABOX, IBOX and SORLIP1. The remaining groups have just a single member; cold regulation is represented by the CGCGBOXAT motif, that of Ca<sup>2+</sup> regulation by ABRRERATCAL, flowering time by CCAATBOX1, ABA/bZIP1 induction by DPBFCOREDCCDC, drought response by MYB2CONSENSUSAT and the MybSt1 binding site (Table 3.1.4). The light regulatory motifs have the highest number of instances within this section of the *DIN3* promoter, a total of four. All other motifs occur just once in the 70bp section (Table 3.1.4). It is worth considering that the first 50bp of the 70bp section, submitted to PLACE to scan for cis-acting regulatory elements, is essential for normal expression of the *DIN3* gene. Once deleted the responsiveness of the gene to environmental stimuli, notably extended darkness, is lost. This is also the section of the promoter analysed by linker scan analysis, the results of which will be considered in §3.5 – 3.6. With this fact in mind, it is reasonable to deduce that there is at least one cis-acting regulatory element listed in Table 3.1.4, which plays an essential role in the normal regulation of *DIN3*. The types of elements found in this section of the promoter could also suggest which cis-acting factors and thereby

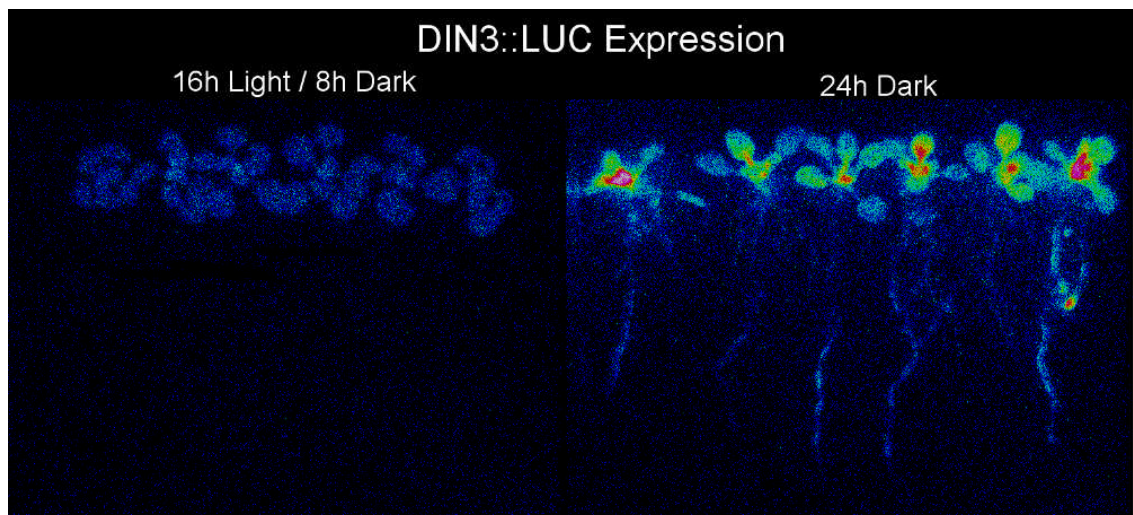
environmental and endogenous stimuli are dominant in the regulation of *DIN3*. Such factors have already been listed, but combining the dominant motifs for the *DIN* genes (Table 3.1.4; figs. 3.1.1/2), light, sugar state and cold temperatures are likely to dominate the regulation of *DIN3*. Light and cold are particularly indicated since only in *DIN3* were all the motifs of the light and cold regulatory groups present (Table 3.1.3).

***DIN3 promoter motifs disrupted by linker scanning mutagenesis lines***

The loci linker scan lines were compared with the loci of cis-acting regulatory motifs identified in the -300 to -230bp using PLACE (Table 3.1.5; fig. 3.1.3). It revealed that the MybSt1 binding site would be disrupted by linker scan lines 3 and 4. Two identified GATA sites (light regulation) are disrupted by scan lines 4 and 11. Linker scan line 5 disrupts the regulatory elements of ABRERATCAL (calcium responsive), CGCGBOXAT (cold) and DPBFCORED CDC (ABA/bZIP) combined (Table 3.1.5; fig. 3.1.3). The drought regulatory element (MYB2CONSENSUSAT) is disrupted by linker scan line 7 (Table 3.1.5; fig. 3.1.3). Scan line 12 was found to disrupt the flowering associated motif (CCAATBOX1) (Table 3.1.5; fig. 3.1.3). Pleasingly this means that every grouping of over-represented cis-acting regulatory motifs identified in these analyses has at least one motif disrupted by linker scan analysis.

### 3.2 Cellular Localisation of *DIN3* expression

To avoid the erroneous interpretation of results, investigation into gene regulation should always be carried out in awareness of the physiological context in which the gene under investigation is expressed. The determination of the cellular expression of *DIN3* was achieved using 17-day-old seedlings expressing a transcriptional fusion of 1000bp of the *DIN3* promoter with the *luc<sup>INT</sup>* gene (Mankin et al., 1997) (*DIN3::LUC*). Seedlings were grown in a 16:8h light:dark cycle then kept in darkness for 24h prior to measurement to guarantee a reliable detection of *DIN3* expression. Luciferin was applied to the seedlings and the resulting luminescence was monitored using a *Photek* photon-counting camera. The luminescence image (fig. 3.2.1: right) obtained represents the expression of *DIN3::LUC* after 24 hours in darkness. This was compared with seedlings that had a further 16:8h light:dark cycle (fig. 3.2.1: left). The seedlings were measured one hour into the apparent 16:8h light:dark cycle.



**Figure 3.2.1 Localisation of *DIN3* expression**

Luminescence image of 17-day-old plants expressing a transcriptional fusion of 1000bp of the *DIN3* promoter with *luc<sup>INT</sup>* (Mankin et al., 1997) (*DIN3::LUC*) following light treatment (left) or 24h darkness (right).

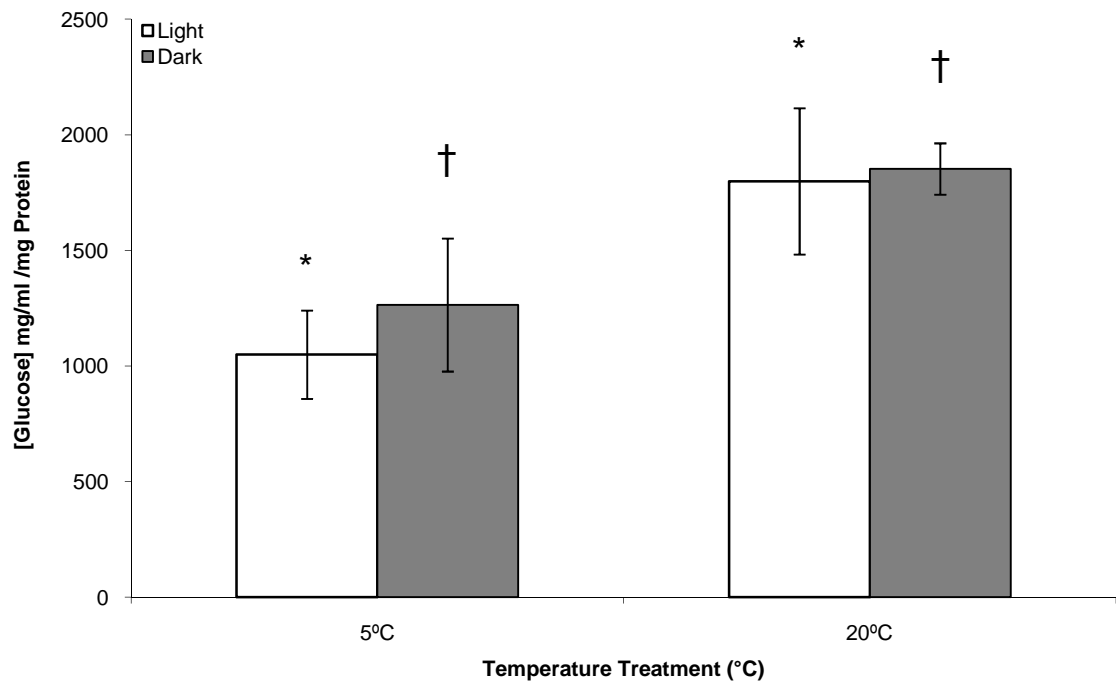
*DIN3::LUC* expression in those seedlings, which received a 24h dark treatment (fig. 3.2.1: right) clearly exceeded *DIN3::LUC* expression in the seedlings which received a 16:8h light:dark cycle (fig. 3.2.1: left). Luminescence was observed in both the aerial parts and roots of the seedlings, with the greatest intensity of luminescence principally restricted to the area of the leaf petiole. These data demonstrate that *DIN3* is expressed in the roots in addition to the aerial or green tissue

### 3.3 Measurement of sugar levels during cold and dark treatment

Given the evidence for the role of sugar state in the regulation of the *DIN* family of genes (Fujiki et al., 2000; Rolland et al., 2006), it is instructive to investigate what effect, if any, the light and temperature treatments had on the sugar levels in the seedlings. Treatments used in these sugar state experiments included three hours of light at 20°C and 5°C and three hours of darkness at 20°C and 5°C. These treatments covered the same conditions used in the latter scanner linker experiments. The seedlings used in these experiments were wild-type Columbia accession 0, grown to 7 days old on 1% MS, 0.8% agar plates under long day conditions (16h:8h; light:dark) at 20°C. Once reaching the necessary age, the treatments were carried out a couple of hours into the light cycle. Three 250mg samples of seedlings which underwent each of the four treatments were harvested and frozen immediately in liquid nitrogen for latter extraction. The samples were extracted according to modified method (Franklin, K., Per. Comm., 2009; §2.9) to that recommended by the *Sigma* glucose/sucrose assay kit. Remaining extracts were also assayed for protein content, against which to standardise the sugar assays.

The concentration of glucose was 1.71 and 1.46 times greater in seedlings treated with three hours in light and darkness at 20°C respectively, compared with those at 5°C (fig. 3.3.1). The difference in glucose concentration between the temperature treatments was significant at the 95% confidence level as determined by a two-tailed *t*-test. This confirmed the significance of the difference between the temperature treatments as indicated by the error bars (fig. 3.3.1).

Interestingly, the difference in glucose concentration between the light and dark treatments within the same temperature treatment was marginal, especially at 20°C. In both cases the error bars overlap and the *t*-test *p* values were greater than 0.05. These results demonstrate that within three hours, glucose levels in 7-day-old seedlings do not vary significantly following a dark treatment compared with those remaining in the light, but that glucose levels decline significantly after three hours at 5°C compared with those staying at 20°C.

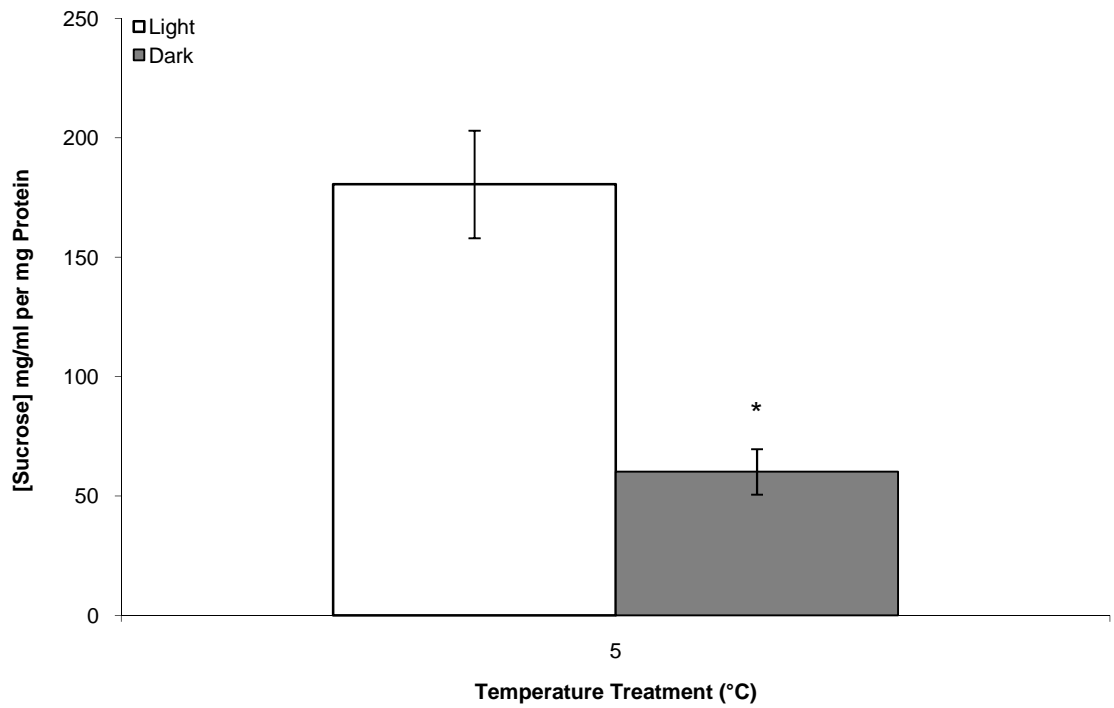


**Figure 3.3.1 Mean glucose levels per mg protein in 7-day-old Col 0 wild-type seedlings following three hours of light or dark treatment at 5 or 20°C.**

Mean glucose levels per mg protein after 3h light (white bars) or dark (grey bars) at 5°C or 20°C. The error bars represent the standard error of the mean. The asterisk (\*) represents a two-tailed t-test carried out between the light-cold and light-ambient treatments  $p=0.027$ ;  $df=2$ ;  $t$  critical= 3.12. The dagger (†) represents a two-tailed t-test carried out between the dark-cold and dark-ambient treatments  $p=0.032$ ;  $df=2$ ;  $t$  critical= 3.12.

Due to spoiled reagents used in the sucrose assay, only the data from the 5°C treated samples were for further analysis. The concentration of sucrose was 3 times greater in the seedlings which remained in the light at 5°C compared with those that spent three in darkness at 5°C (fig. 3.3.2). This difference in the means of these two samples is more than 2 S.E.M. from each other and so can be considered significant.

Without at least one sample of sucrose concentration at 20°C as a control comparison against which to compare the effects of the three hour 5°C treatment on sucrose concentration, it is tenuous to draw any profound conclusions from these results. The implications of these results will be explored in greater detail in the discussion.



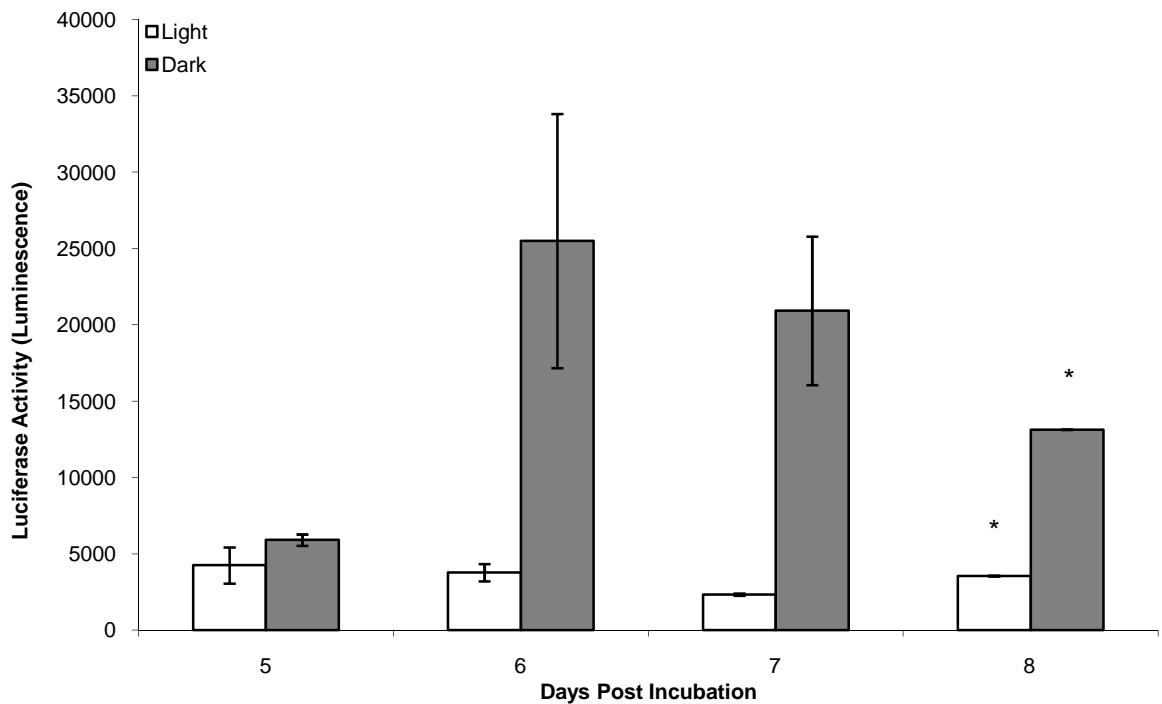
**Figure 3.3.2 Mean sucrose concentration per mg protein in 7-day-old Col 0 wild-type seedlings treated with three hours of light or dark at 5°C.**

Mean sucrose concentration per mg protein after 3h light (white bars) or dark (grey bars) at 5°C. The error bars represent the standard error of the mean. The asterisk (\*) indicates that the dark 5°C average was  $\geq 2$  S.E.M. from the light 5°C; n=3.

### 3.4 Analysis of *DIN3* expression over developmental stages

Given the variability seen in the quality of seedling growth between transformant lines, it seemed instructive to assay the expression patterns of *DIN3* and *DIN3::LUC* in order to determine what, if any, effect developmental stage had. To mimic the range of growth quality, Columbia accession 0 (Col 0) seedlings, expressing a transcriptional fusion (*DIN3::LUC*) of 300bp of the *DIN3* promoter with the *LUC<sup>INT</sup>* (Mankin et al., 1997) gene were grown under long days (16:8h light:dark) to 5, 6, 7 and 8 days post germination then subjected to six-hour treatments in the light or darkness at 20°C, the same used on transformant lines, then analysed by luminometry and qRT-PCR to determine the expression patterns of *DIN3* and *DIN3::LUC* genes.

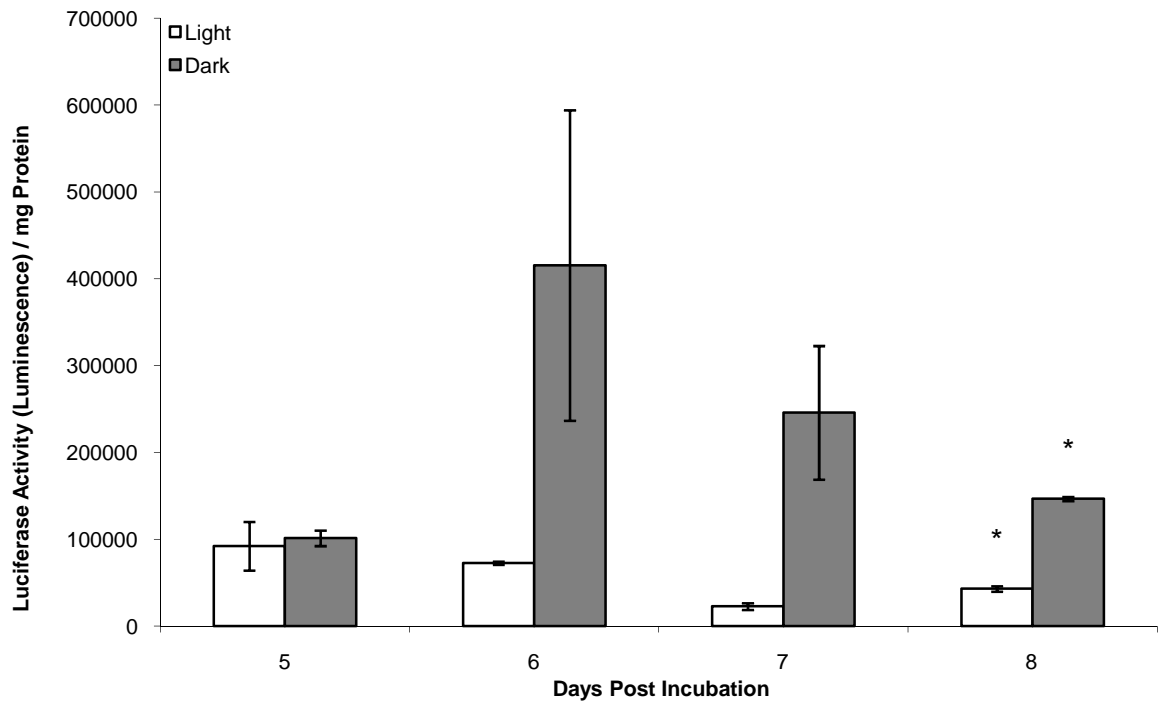
At every stage of seedling development studied, the *DIN3::LUC* expression, as determined by luciferase luminometry, was greater in those seedlings which received a 6h dark treatment. The *DIN3::LUC* expression was greatest in the 6-day-old seedlings, however the difference between the light and dark *DIN3::LUC* expression was greatest amongst the 7-day-old seedlings, with *DIN3::LUC* expression the dark treated seedlings 8.97 times greater than those remaining in the light. The light/dark difference between the *DIN3::LUC* expression was greatest in the 5-day-old seedlings. A two-tailed *t*-test revealed that only the 8-day-old seedlings had *DIN3::LUC* expression significantly different at the 95% confidence level between the light and dark treatments (fig. 3.4.1). As was the procedure in all luciferase luminometry assays, 10 seedlings were used in each sample. This was the only method used ensure that the amount of tissue remained constant between samples. However with variability in growth, the efficacy such a method of control is questionable. Therefore to ascertain the controls efficacy, the protein content of the samples was determined.



**Figure 3.4.1 *DIN3::LUC* expression across developmental stages**

Mean *DIN3::LUC* expression, determined by luminometry, in five, six, seven and eight-day-old Col 0 seedlings expressing a transcriptional fusion of 300bp of the *DIN3* promoter with the *LUC<sup>INT</sup>* gene (Mankin et al., 1997) following six-hour light or dark treatment. Assays used two samples of 10 seedlings. Error bars represent the standard error of the mean. The asterisk (\*) indicates those light and dark treatments whose data are significantly different from each other as determined by a two-tailed *t*-test (*t*-test result for 8-day-old seedlings:  $p=0.001$ ;  $df=1$ ;  $t_{critical}=12.71$ .)

The determination of the protein content of the samples the luciferase luminometry data (fig. 3.4.1) was corrected, thereby accounting for variation of seedling size in the samples which could have caused the pattern seen from the luciferase luminometry alone (fig. 3.4.1). The *DIN3::LUC* expression pattern across the development stages, seen with the luminometry data alone (fig. 3.4.1), remains intact when the data is corrected for the individual protein content in each sample (fig. 3.4.2). Slight differences, are noticeable; the difference between the highest *DIN3::LUC* expression, that of the 6-day-old seedlings, and the next, that of the 7-day-old seedlings, is more pronounced once corrected for protein, increasing from 1.22 to 1.69 times. The magnitude difference between light and dark *DIN3::LUC* expression in the 8-day-old seedlings is smaller, declining from 3.7 times to 3.4 times once corrected for protein. However the *t*-test suggests this difference is more significant, at the 95% confidence level, once corrected for protein (fig. 3.4.2).



**Figure 3.4.2 *DIN3::LUC* expression per mg of protein across developmental stages**

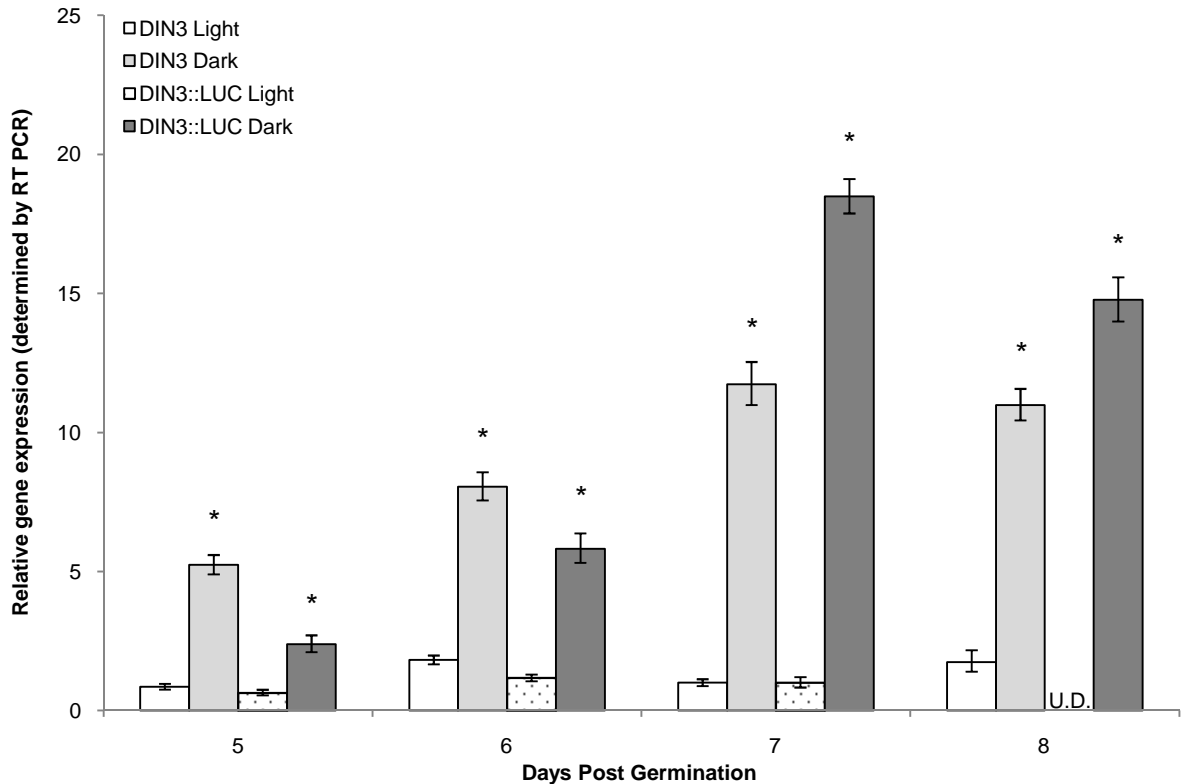
Mean *DIN3::LUC* activity, determined by luminometry and standardised against mg protein, in five, six, seven and eight day old Col 0 seedlings expressing a transcriptional fusion of 300bp of the *DIN3* promoter with the *LUC<sup>INT</sup>* gene (Mankin et al., 1997) following six-hour light or dark treatment. Error bars represent the standard error of the mean. The asterisk indicates those light and dark treatments whose data are significantly different from each other as determined by a two-tailed *t*-test (*t*-test result for 8-day-old seedlings:  $p=0.0003$ ;  $df=1$ ;  $t$  critical= 12.71.)

Analysis by qRT-PCR is internally corrected for variation in template (cDNA) amounts to an endogenous reference gene (in most cases *PEX4*), whose expression is not significantly affected by the experimental treatments. The qRT-PCR provides a direct measurement of relative gene expression; it additionally permits the measurement of the *DIN3* and *DIN3::LUC* expression following the dark cold treatments. Although qRT-PCR is corrected for variation in sample quality, it is still instructive to determine whether development stage *per se*, rather than the sample quality, affects *DIN3* and *DIN3::LUC* expression patterns.

At every stage in development the *DIN3* expression was greater amongst the 6h dark treated than those treated in light for six-hours (fig. 3.4.3). This observation holds true for the *DIN3::LUC* expression, whose pattern matches that of *DIN3* at every developmental stage.

The difference in light/dark *DIN3* expression was least amongst the 6-day-old seedlings, at 4.42 times, and was greatest amongst the 7-day-old seedlings at 11.74 times. The magnitude of dark induction increase with developmental age, from 5.24 times the 7-day-old light sample to a peak at 7 days old at 11.74 times. The increase *DIN3::LUC*

expression with developmental and peak at 7 days old mirrored that seen in the *DIN3* expression. The light/dark difference in *DIN3::LUC* expression was least in the 5-day-old seedlings, rather than those of 6 days in age, as seen with *DIN3* expression. The *DIN3::LUC* light/dark expression difference was greatest in the 7-day-old seedlings, mirroring that of *DIN3* expression. Although with no determinable *DIN3::LUC* expression for 8-day-old light treated seedlings, the light/dark difference can only be assumed to be at least 14.76 times as compared with the 18.49 times in the 7-day-old seedlings (fig. 3.4.3). The greatest light/dark expression difference as determined by qRT-PCR to be within the 7-day-old seedlings (fig. 3.4.3) matched that determined by luminometry (figs 3.4.1/2). However the peak *DIN3::LUC* expression determined by qRT-PCR to be within the 7-day-old seedlings (fig. 3.4.3) was not mirrored by the results from the luminometry (figs. 3.4.1/2), which indicated peak *DIN3::LUC* expression to be within the 6-day-old seedlings.



**Figure 3.4.3 Relative *DIN3* and *DIN3::LUC* expression across developmental stages**

Relative *DIN3* and *DIN3::LUC* expression levels in Col 0 seedlings expressing a transcriptional fusion of 300bp of the *DIN3* promoter with the *LUC<sup>NT</sup>* gene (Mankin et al., 1997) (standardised against *PEX4* expression) between 5 and 8 days post germination and after a 6h light or dark treatment at 20°C. Expression levels are relative to the light sample for the 7-day-old seedlings. U.D. = Expression Undetermined i.e. so low as beyond the limits of detectability. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient data set. Error bars represent the Student's *t*-distribution maxima and minima, d.f.= 7.

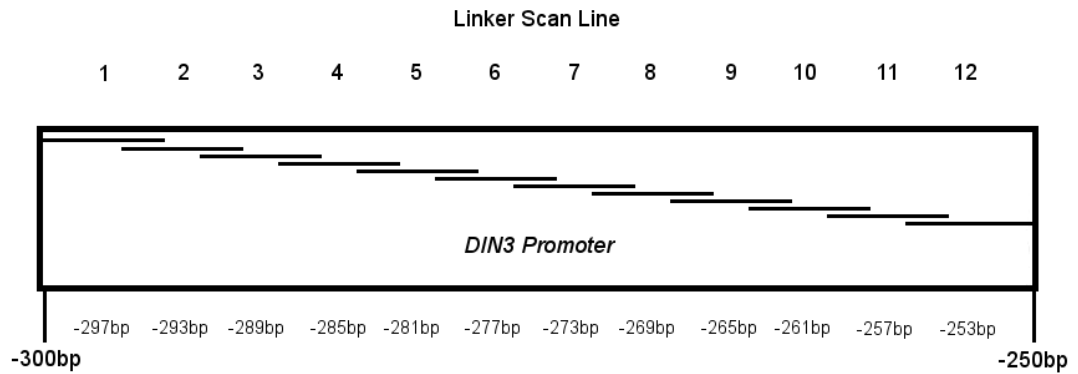
These analyses of the effect of developmental stage on the expression of *DIN3* / *DIN3::LUC* demonstrated that there was maintenance in the overall pattern of *DIN3* expression, which is recognised to be an increase or induction of *DIN3* expression after a period of extended darkness (Fujiki et al., 2000). However there were differences in both peak *DIN3* expression and the magnitude of the difference between the light and dark treatments across the seedling age range. Luminometry indicated that the peak *DIN3::LUC* expression is found at the 6-day-old stage, whereas qRT-PCR determined the peak *DIN3* and *DIN3::LUC* expression to be at the 7-day-old stage. Both methods produced results indicating that the seedlings which exhibited greatest difference between their light and their dark *DIN3* / *DIN3::LUC* expression, were 7 days old.

### 3.5 Linker Scan Analysis of 50bp of 300bp minimal *DIN3* promoter

Previous research (Knight, H., personal communication), revealed that the 300bp of the *DIN3* promoter upstream of the ATG start codon was essential for responsiveness of *DIN3* to darkness, reported previously by (Fujiki et al., 2000)). This result was derived from promoter deletion analysis, constructing a number of successively smaller truncations of the *DIN3* promoter fused to luciferase gene (*luc<sup>INT</sup>*) (Mankin et al., 1997) as a reporter. The *luc<sup>INT</sup>* gene has an artificial intron to ensure that observed luminescence is due to *in planta* expression rather than ectopic bacterial expression. When the promoter was reduced to 250bp it became non-responsive, making the 300bp the minimum functional promoter of *DIN3*. Therefore it was hypothesised that the 50bp from the 5' end of the 300bp *DIN3* promoter contained cis-acting elements without which no induction of *DIN3* was possible.

To investigate the essential 50bp more fully, transgenes were assembled from the 300bp *DIN3::LUC* promoter fusion and analysed by the linker-scanning method of mutagenesis (McKnight & Kingsbury, 1982). This required the creation of 12 individual linker scan lines which had the sequence of the *EcoRI* site (GAATTC) substituted for 6bp of the native 300bp *DIN3* promoter sequence. Starting from -300bp upstream of the start codon, the substitutions in subsequent linker scan lines overlapped, by 2bp, relative locus of the substitution of the preceding linker-scanning line (henceforth scan lines), so that the 12 lines covered the entire 50bp (fig. 3.5.1). Substitution mutagenesis ensures maintenance of an identical frame to the original sequence. Severally independently transformed lines expressing the unmodified 300bp of the *DIN3::LUC* fusion (hereafter *DIN3* promoter reporting lines) were used for controls for the experimental method, but also as controls against which to compare the scan lines since their expression patterns should mirror that of the native *DIN3*. When the control and scan lines were originally transformed (§2.4 Plant Transformation) multiple independent transformant lines came through the selection screen expressing the same transgene. These are replicates in themselves, which demonstrate the consensus of the effect of the promoter modification. The mode of transformation is such that the eventual transgene locus is random and will be different in each transformant line. If the transgene locus is not significant, one would anticipate that the expression pattern will be the same, if not of the same magnitude of expression, creating a consensus. Initially the effects of the

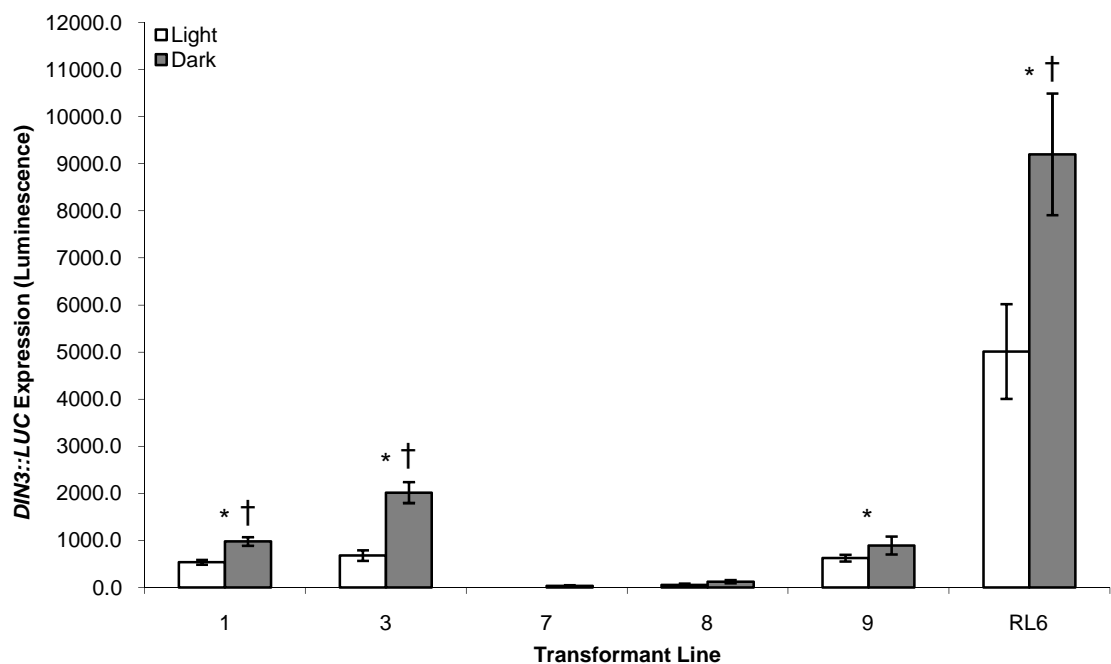
promoter modifications were analysed by applying luciferin to the lysate of 10, 7-day-old seedlings of the various scan and control lines treated either with six-hours of light or darkness at 20°C and measuring the resulting luminescence via a luminometer. It is the results of such analyses which will be considered in this section.



**Figure 3.5.1 Loci of linker-scanning mutations in the promoter of *DIN3::LUC* of the scan lines**

The loci of the linker-scanning mutations – EcoRI (*GAATTC*) substitutions (black lines) – shown at their relative positions in the -300bp to -250bp portion *DIN3::LUC* promoter upstream of the ATG. The numbers (base-pairs) indicate the locus of the third base of the EcoRI substitution. The second linker-scanning substitution mutation overlaps the locus of the previous by two bases pairs, providing optimal disruption of any cis-acting regulatory motifs present. Only one of the linker-scanning mutations is present in each linker scan line concurrently.

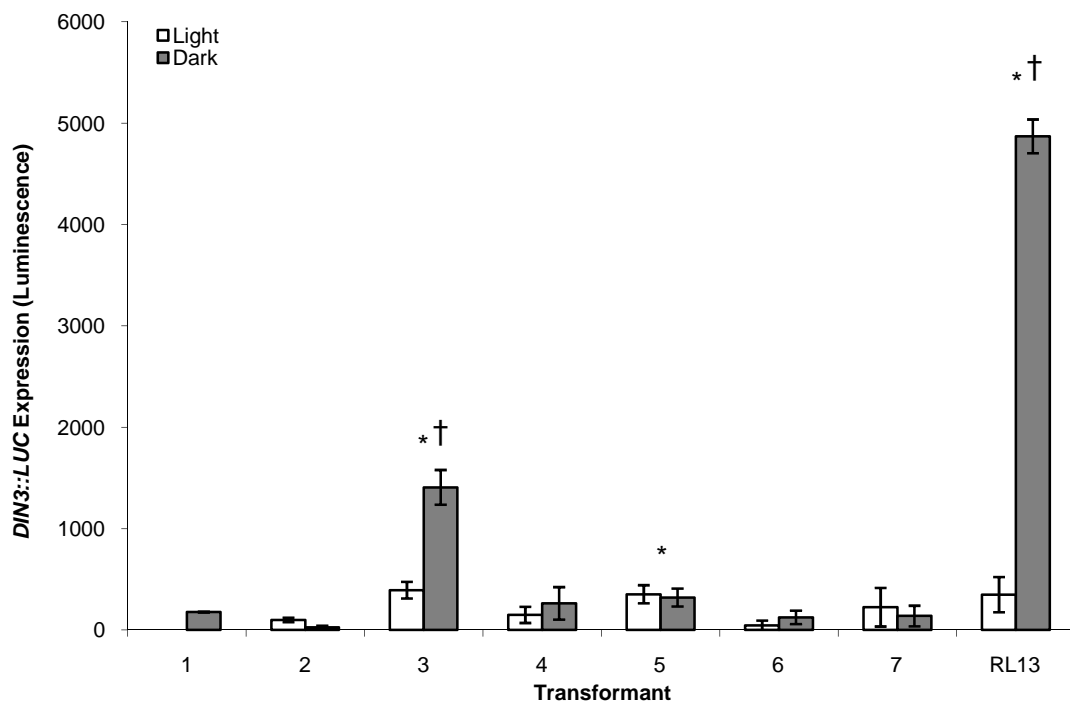
Seedlings of transformant lines 1 and 3 of Scan Line 1 exhibited an increase in *DIN3::LUC* expression following a 6h dark treatment compared to expression after the 6h light treatment (fig. 3.5.2). The 6h dark treatment did not elicit detectable levels of *DIN3::LUC* expression in lines 7 and 8; line 9, whilst detectable for *DIN3::LUC* expression, did not exhibit significantly different light/dark *DIN3::LUC* expression (fig. 3.5.2). Overall, the pattern of *DIN3::LUC* expression appears to match that of the native *DIN3* expression, as seen in *DIN3* promoter reporting line 6, whereby dark treated seedlings display *DIN3::LUC* expression levels significantly above their light treated siblings. The modified *DIN3::LUC* expressed by Scan Line 1 seedlings appears unaffected when compared with unmodified *DIN3::LUC* reporter expression (RL6), that is it has retains the characteristic expression (fig. 3.4.3). The Scan Line 1 mutation changes GTGATG of the native sequence to GAATTC; no known cis-acting motifs in part or entirety were detected within the 6 base-pairs of the EcoRI substitution.



**Figure 3.5.2 Mean *DIN3::LUC* expression in 7-day-old Scan Line 1 seedlings following six-hour light and dark treatment.**

Mean *DIN3::LUC* expression, determined by luminometry, in 7-day-old Scan Line 1 seedlings following either 6h light (white) or dark (grey) treatment. The error bars represent the standard error of the mean. *DIN3* promoter reporting line 6 (RL6) was included as a comparison; this line expresses the unmodified 300bp of the *DIN3* promoter fused to luciferase. These data are not normally distributed as can be seen by the larger S.E.M. for those data with greater expression. These data were transformed by a  $\log_{10}(x+1)$  transformation and compared with the background luminescence. The asterisk (\*) indicates those data sets which were at least 2 S.E.M. from the background. The dagger (†) indicates those transformant lines whose light/dark *DIN3::LUC* expression was >2 S.E.M. apart.

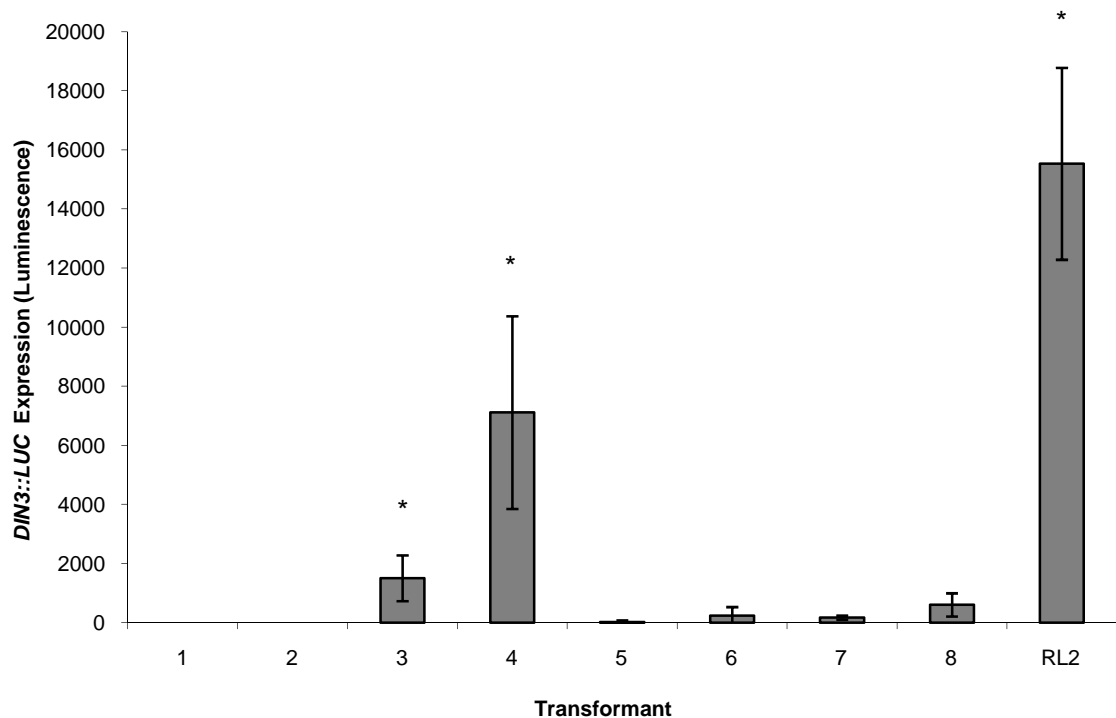
Seedlings of transformant line 3 of Scan Line 2 exhibited an increased *DIN3::LUC* expression following a 6h dark treatment over a 6h light treatment (fig. 3.5.3); lines 1, 2, 4 and 7 did not exhibit detectable levels of *DIN3::LUC*; line 5, whilst detectable for *DIN3::LUC* expression, did not exhibit a significant difference in light/dark *DIN3::LUC* expression (fig. 3.5.3). Since only one out of two lines, which expressed *DIN3::LUC* at detectable levels exhibited the characteristic induction of *DIN3::LUC* following a 6h dark treatment, as seen in the *DIN3* promoter reporting line, the effect of the modification expressed Scan Line 2 cannot be determined. The Scan Line 2 mutation changes TGTACA of the native sequence to GAATTC; no known cis-acting motifs in part or entirety were detected within the 6 base-pairs of the EcoRI substitution.



**Figure 3.5.3 Mean *DIN3::LUC* expression in 7-day-old Scan Line 2 seedlings following six-hour light and dark treatment**

Mean *DIN3::LUC* expression, determined by luminometry, in 7-day-old Scan Line 2 seedlings following either 6h light (white) or dark (grey) treatment. The error bars represent the standard error of the mean. *DIN3* promoter reporting line 13 (RL13) was included as a comparison; this line expresses the unmodified 300bp of the *DIN3* promoter fused to luciferase. These data are not normally distributed as can be seen by the larger S.E.M. for those data with greater expression. These data were transformed by a  $\log_{10}(x+1)$  transformation and compared with the background luminescence. The asterisk (\*) indicates those data sets which were at least 2 S.E.M. from the background. The dagger (†) indicates those transformant lines whose light/dark *DIN3::LUC* expression was >2 S.E.M. apart.

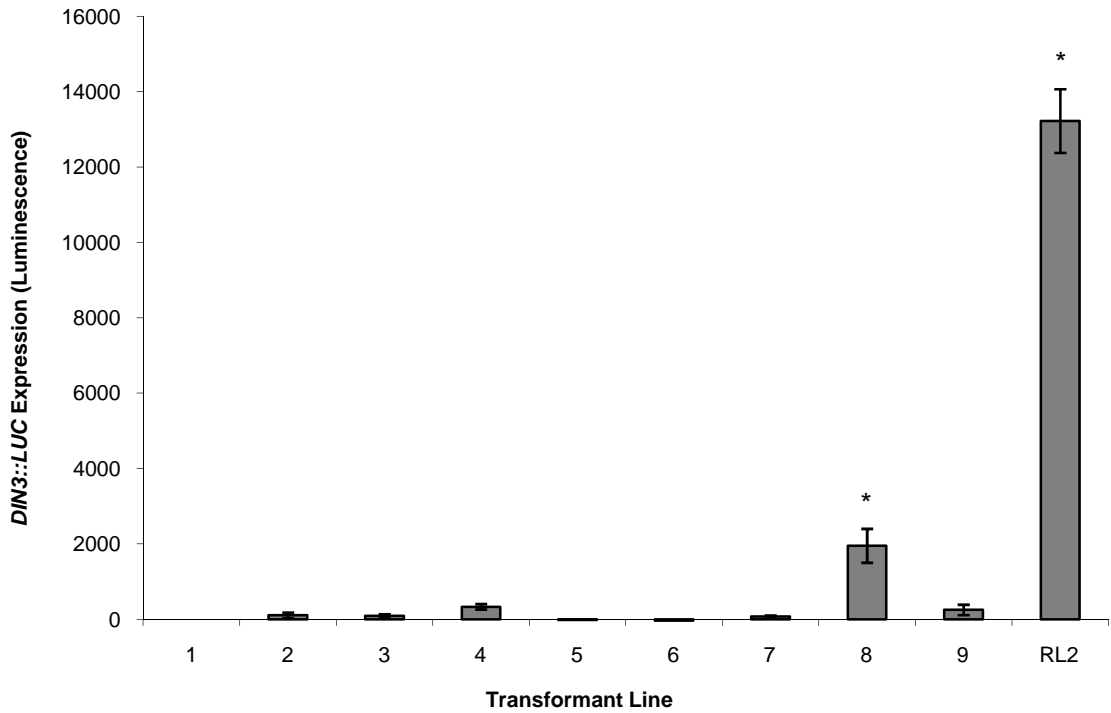
Seedlings of Scan Line 3 were treated with a 6h light or dark treatment, however it was found that the differences between the *DIN3::LUC* expression in the light and dark treatments were not significant. The experiment was repeated (results herein) with a 6h dark treatment alone. Seedlings of transformant lines 3 and 4 of Scan Line 3 exhibited *DIN3::LUC* expression at detectable levels following the 6h dark treatment (fig. 3.5.4). Lines 1, 2, 5, 6, 7 and 8 did not exhibit detectable levels of *DIN3::LUC* expression. Since only two out of eight lines exhibited *DIN3::LUC* expression at detectable levels and none the characteristic induction of *DIN3::LUC* following a 6h dark treatment, as seen in the *DIN3* promoter reporting line, the effect of the mutation in the version of *DIN3::LUC* expressed by Scan Line 3, can be said to have disrupted *DIN3::LUC* expression compared to native *DIN3* expression. The Scan Line 3 mutation changes CATGAG of the native sequence to GAATTC; a MYBST1 cis-acting motif (CGATA) was detected within the 6 base-pairs of the EcoRI substitution.



**Figure 3.5.4 Mean *DIN3::LUC* expression in 7-day-old Scan Line 3 seedlings following six-hour dark treatment.**

Mean *DIN3::LUC* expression, determined by luminometry, in 7-day-old Scan Line 3 seedlings following a 6h dark treatment. The error bars represent the standard error of the mean. *DIN3* promoter reporting line 2 (RL2) was included as a comparison; this line expresses the unmodified 300bp of the *DIN3* promoter fused to luciferase. These data are not normally distributed as can be seen by the larger S.E.M. for those data with greater expression. These data were transformed by a  $\log_{10}(x+1)$  transformation and compared with the background luminescence. The asterisk (\*) indicates those data sets which were at least 2 S.E.M. from the background.

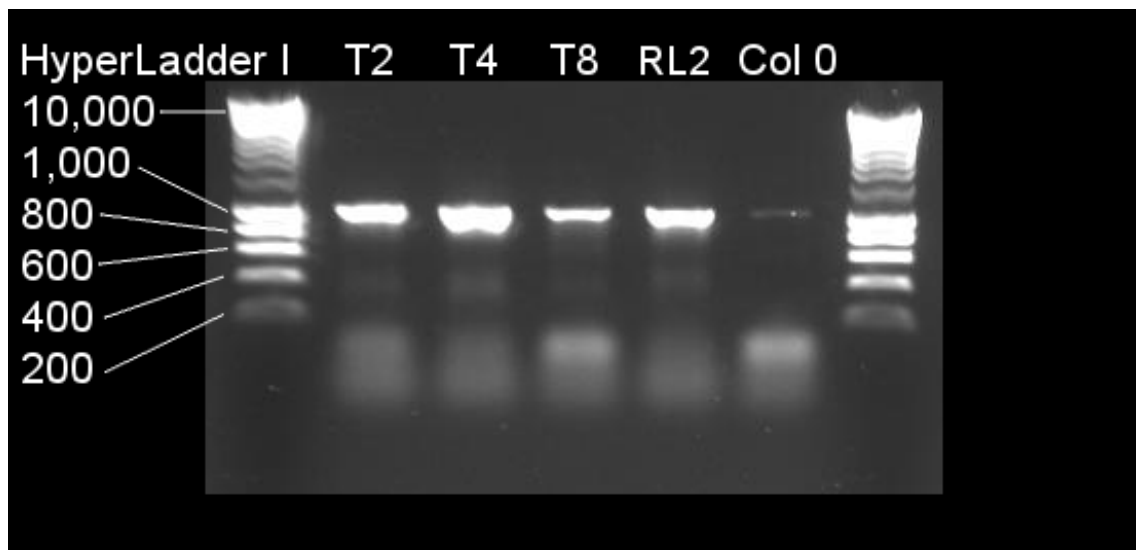
Seedlings of Scan Line 4 were treated with a 6h light or dark treatment, however it was found that the differences between the *DIN3::LUC* expression of the light and dark treatments were not significant. The experiment was repeated (results herein) with a 6h dark treatment alone. Seedlings of transformant line 8 of Scan Line 4 exhibited detectable levels *DIN3::LUC* expression following a 6h dark treatment (fig. 3.5.5) All other lines, excluding *DIN3* promoter reporting line 2, did not exhibit detectable levels of *DIN3::LUC* expression . Since only one out of nine lines exhibited *DIN3::LUC* expression at detectable levels and none the characteristic induction of *DIN3::LUC* following a 6h dark treatment, as seen in the *DIN3* promoter reporting line, the effect of the mutation in the version of *DIN3::LUC* expressed by Scan Line 4, can be said to have disrupted *DIN3::LUC* expression compared to native *DIN3* expression. The Scan Line 4 mutation changes AGGATA of the native sequence to GAATTC; the MYBST1 (CGATA), GATABOX (GATA) and DPBFCOREDCDC (CGCGTGT) cis-acting motifs in part or entirety were detected within the 6 base-pairs of the EcoRI substitution.



**Figure 3.5.5 Mean *DIN3::LUC* expression in 7-day-old Scan Line 4 seedlings following six-hour dark treatment**

Mean *DIN3::LUC* expression, determined by luminometry, in 7-day-old Scan Line 4 seedlings following 6h dark treatment. The error bars represent the standard error of the mean. *DIN3* promoter reporting line 2 (RL2) was included as a comparison this line expresses the unmodified 300bp of the *DIN3* promoter fused to luciferase. These data are not normally distributed as can be seen by the larger S.E.M. for those data with greater expression. These data were transformed by a  $\log_{10}(x+1)$  transformation and compared with the background luminescence. The asterisk (\*) indicates those data sets which were at least 2 S.E.M. from the background.

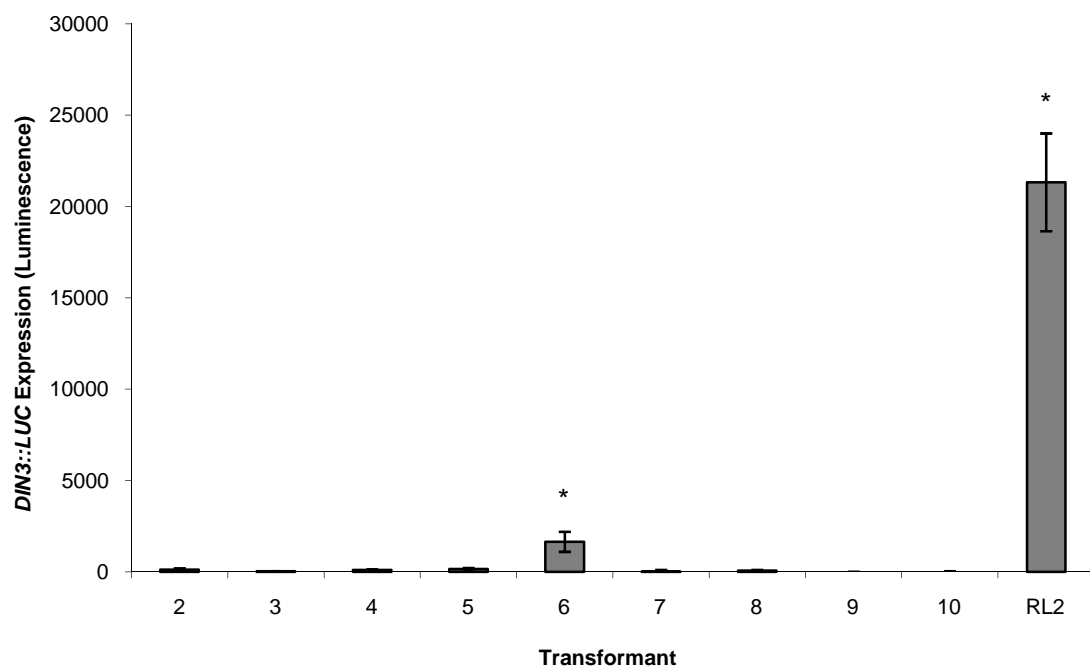
Seedlings of Scan Line 5 were treated with a 6h light or dark treatment, however it was found that the difference between the *DIN3::LUC* expression of the light and dark treatments were not significant. A second experiment whereby seedlings of Scan Line 5 were treated with six-hours of darkness found no detectable lines of *DIN3::LUC* in any of the transformant lines. The absence of detectable *DIN3::LUC* expression begged the question: whether the transformants of Scan Line 5 were genuinely possessed the transgene in their genomes. To confirm this, a PCR was set up using primers to the *luc<sup>INT</sup>* gene and genomic DNA, as the template, from seedlings of transformants 2, 4 and 8, in addition to *DIN3* promoter reporting line 2 and Columbia 0 wild-type; the former serving as positive and the latter as negative controls. If transformants 2, 4 and 8 of Scan Line 5 were genuine, then a band similar to that of the positive control, *DIN3* promoter reporting line 2, should be present in the gel. The PCR confirmed that the presence of *DIN3::LUC* in the genomes of transformants 2, 4 and 8 of Scan Line 5, therefore these were genuine transformants (fig. 3.5.6). These results indicate that expression of the modified version of *DIN3::LUC* found in the genome of Scan Line 5 seedlings has been severely inhibited by the linker-scanning mutation which changed TACGGCG of the native sequence to GAATTC affecting the cis-acting motifs of ABRERATCAL (ACGCGTG), CGCGBOXAT (ACGCGT) and DPBFCORED CDC (CGCGTGT) by disrupting part of or their entire sequence.



**Figure 3.5.6 Gel electrophoresis of PCR confirming presence of *DIN3::LUC* in Scan Line 5.**

Figure 3.5.6 Image of a 1% agarose electrophoretic gel of PCR products using primers to the *LUC* gene. The templates for the PCR were samples of extracted genomic DNA of Scan Line 5 transformant Lines 2 (T2), 4 (T4) and 8 (T8) compared alongside *DIN3* promoter reporting line 2 (RL2) and Columbia 0 wild-type (Col 0). Bioline HyperLadder I was used as a size marker (numbers denote base-pairs).

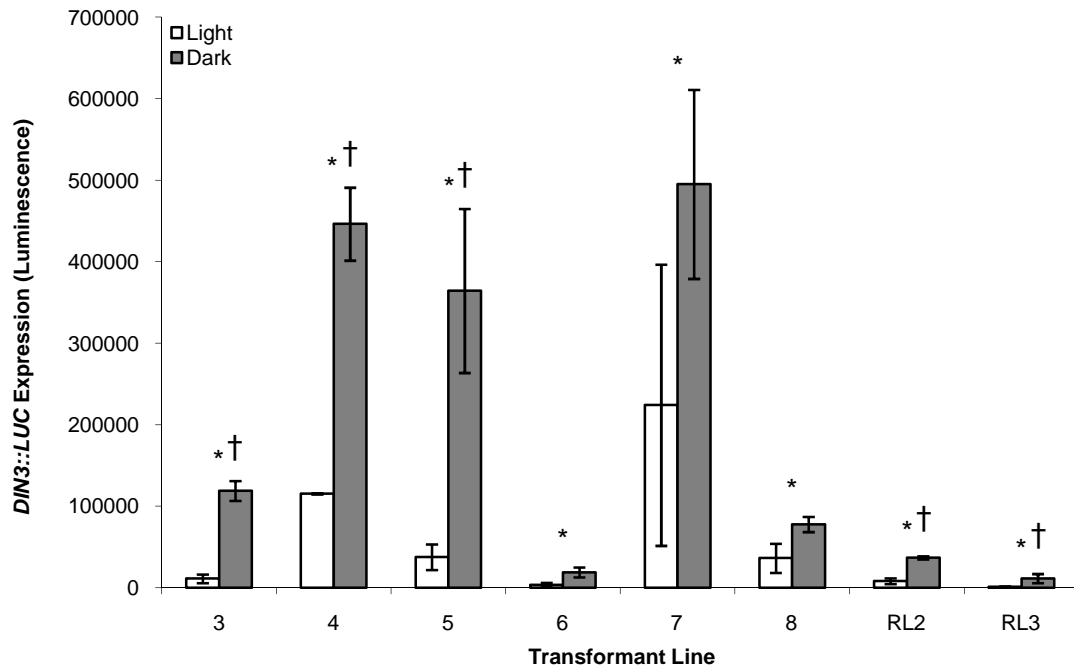
Seedlings of Scan Line 6 were treated with a 6h light or dark treatment, however it was found that the differences between the *DIN3::LUC* expression of the light and dark treatments were not significant. A second experiment found that seedlings of transformant line 6 of Scan Line 6 exhibited detectable levels of *DIN3::LUC* expression following a 6h dark treatment (fig. 3.5.7). All other lines, excluding *DIN3* promoter reporting line 2, did not exhibit *DIN3::LUC* expression at detectable levels. Since only one out of nine lines expressed *DIN3::LUC* at detectable levels and none exhibited the characteristic induction of *DIN3::LUC* following a 6h dark treatment, the effect of the mutation in the version of *DIN3::LUC* expressed by Scan Line 6 can be said to have disrupted *DIN3::LUC* expression compared to native *DIN3* expression. The Scan Line 6 mutation changes CGTGTA of the native sequence to GAATTC; ABRERATCAL (ACGCGTG), CGCGBOXAT (ACGCGT) cis-acting motifs in part or entirety were detected within the 6 base-pairs of the EcoRI substitution.



**Figure 3.5.7 Mean *DIN3::LUC* expression in 7-day-old Scan Line 6 seedlings following six-hour light and dark treatment**

Mean *DIN3::LUC* expression, determined by luminometry, in 7-day-old Scan Line 6 seedlings following 6h dark treatment. The error bars represent the standard error of the mean. *DIN3* promoter reporting line 2 (RL2) was included as a comparison; this line expresses the unmodified 300bp of the *DIN3* promoter fused to luciferase. These data are not normally distributed as can be seen by the larger S.E.M. for those data with greater expression. These data were transformed by a  $\log_{10}(x+1)$  transformation and compared with the background luminescence. The asterisk (\*) indicates those data sets which were at least 2 S.E.M. from the background.

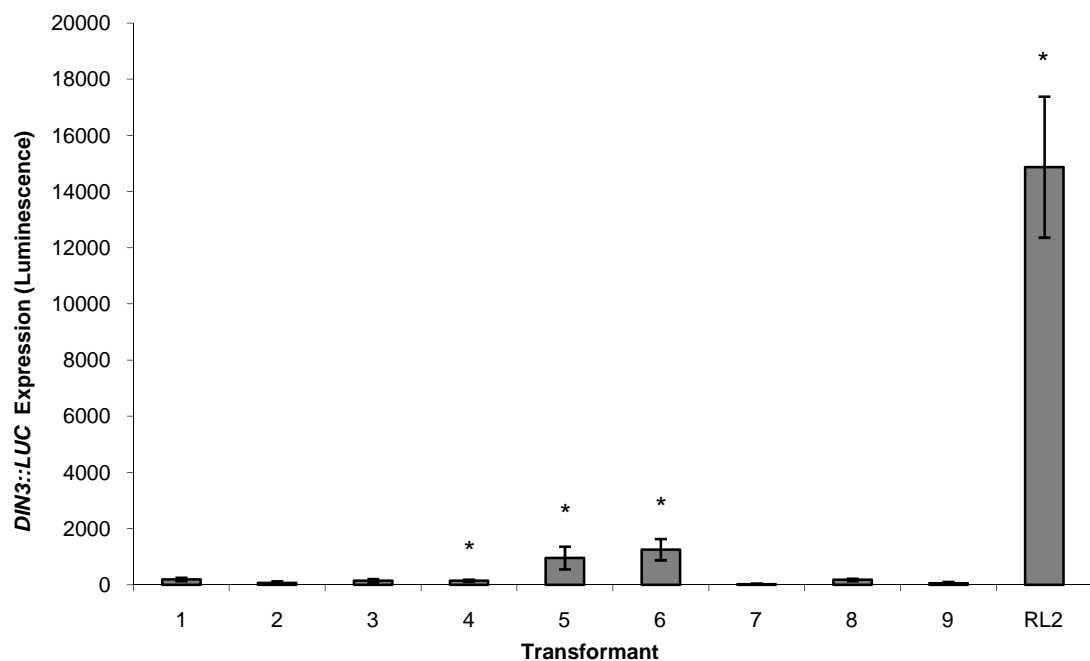
All transformant lines of Scan Line 7 exhibited *DIN3::LUC* expression which was greater following the 6h dark treatment compared with the 6h light treatment (fig. 3.5.8). However with transformant lines 6, 7 and 8 this difference was not significant (fig. 3.5.8). Amongst all the transformant lines and *DIN3* promoter reporting lines, *DIN3::LUC* was expressed at detectable levels following 6h dark treatment. It should also be noted that except for transformant line 6, all other lines demonstrated a much greater *DIN3::LUC* expression than *DIN3* promoter reporting line 2 (up to 13.45 times greater), which exhibits the highest level of *DIN3::LUC* expression amongst the reporting lines. Even the *DIN3::LUC* expression levels of Scan Line 7, following the 6h light treatment, were greater than the *DIN3* promoter reporting line 2 dark average. Overall, the pattern of *DIN3::LUC* expression seems to match that of *DIN3* promoter reporting lines 2 and 3. However the magnitude of the *DIN3::LUC* expression amongst Scan Line 7 is unparalleled, either amongst the other Scan Lines or the *DIN3* promoter reporting lines. The mutation in the version of *DIN3::LUC* expressed by the Scan Line 7 seedlings did not disrupt or eliminate the characteristic dark induction seen in the native *DIN3* expression. However, whether the modification of the *DIN3::LUC* expressed by Scan Line 7 is the cause of the high expression levels observed is a matter for discussion. The Scan Line 7 mutation changes TACCGT of the native sequence to GAATTC; a MYB2CONSENSUSAT cis-acting motif (CCGTTG) in part or entirety were detected within the 6 base-pairs of the EcoRI substitution.



**Figure 3.5.8 Mean *DIN3::LUC* expression in 7-day-old Scan Line 7 seedlings following six-hour light and dark treatment**

Mean *DIN3::LUC* expression, determined by luminometry, in 7-day-old Scan Line 7 seedlings following either 6h light (white) or dark (grey) treatment. The error bars represent the standard error of the mean. *DIN3* promoter reporting lines 2 and 3 (RL2/3) were included for comparison; these lines express the unmodified 300bp of the *DIN3* promoter fused to luciferase. These data are not normally distributed as can be seen by the larger S.E.M. for those data with greater expression. These data were transformed by a  $\log_{10}(x+1)$  transformation and compared with the background luminescence. The asterisk (\*) indicates those data sets which were at least 2 S.E.M. from the background. The dagger (†) indicates those transformant lines whose light/dark *DIN3::LUC* expression was >2 S.E.M. apart.

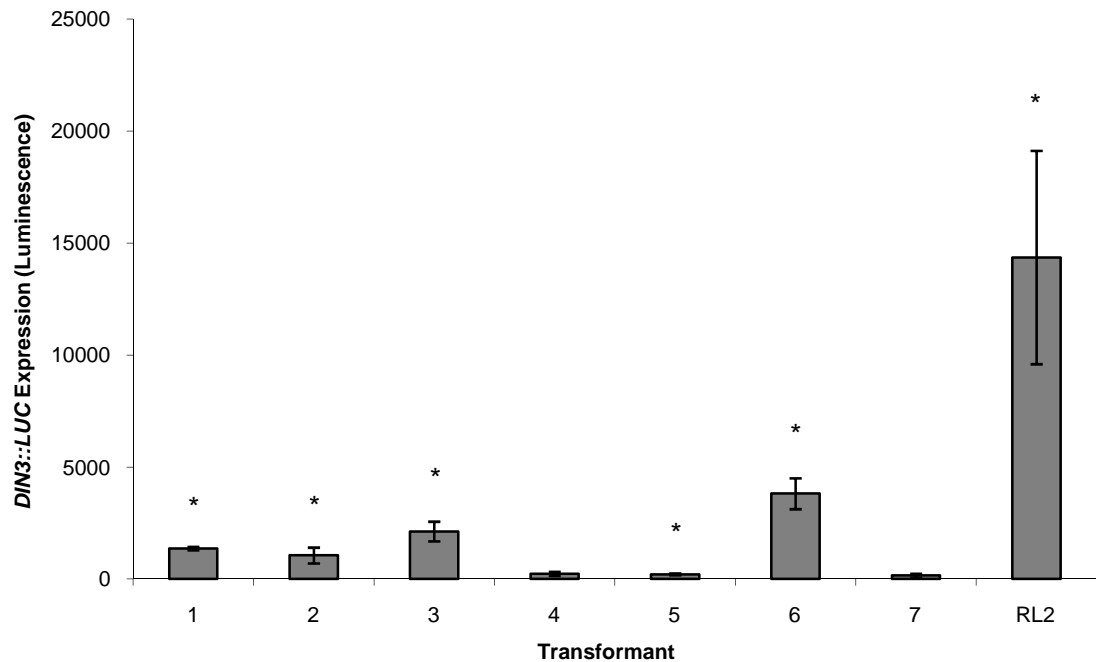
Seedlings of Scan Line 8 were treated with a 6h light or dark treatment, however it was found that the differences between the *DIN3::LUC* expression of the light and dark treatments were not significant. A second experiment found that following a 6h dark treatment, seedlings of transformant lines 4, 5 and 8 of Scan Line 8 exhibited *DIN3::LUC* expression at detectable levels (fig. 3.5.9). All other lines, excluding *DIN3* promoter reporting line 2, did not exhibit *DIN3::LUC* expression at detectable levels. Since only three out of nine lines expressed *DIN3::LUC* at detectable levels and none exhibited the characteristic induction of *DIN3::LUC* following a 6h dark treatment, the effect of the mutation in the version of *DIN3::LUC* expressed by Scan Line 8 can be said to have disrupted *DIN3::LUC* expression compared to native *DIN3* expression. The Scan Line 8 mutation changes GTTGAT of the native sequence to GAATTC; a MYB2CONSENSUSAT cis-acting motif (CCGTTG) was detected within the 6 base-pairs of the EcoRI substitution.



**Figure 3.5.9 Mean *DIN3::LUC* expression in 7-day-old Scan Line 8 seedlings following six-hour dark treatment**

Mean *DIN3::LUC* expression, determined by luminometry, in 7-day-old Scan Line 8 seedlings following either 6h dark treatment. The error bars represent the standard error of the mean. *DIN3* promoter reporting line 2 (RL2) was included as a comparison this line expresses the unmodified 300bp of the *DIN3* promoter fused to luciferase. These data are not normally distributed as can be seen by the larger S.E.M. for those data with greater expression. These data were transformed by a  $\log_{10}(x+1)$  transformation and compared with the background luminescence. The asterisk (\*) indicates those data sets which were at least 2 S.E.M. from the background.

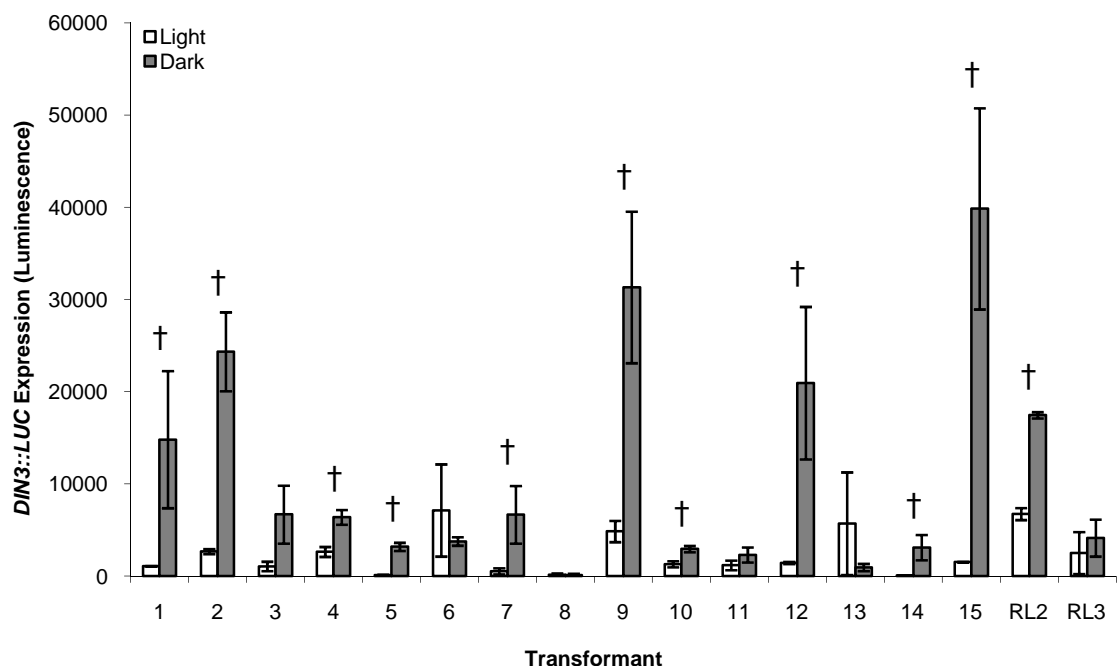
Seedlings of Scan Line 9 were treated with a 6h light or dark treatment, however it was found that the differences between the *DIN3::LUC* expression of the light and dark treatments were not significant. A second experiment found that seedlings of transformant lines 1, 2, 3, 5 and 6 of Scan Line 9 expressed *DIN3::LUC* at detectable levels following a 6h dark treatment (fig. 3.5.10). All other lines, excluding *DIN3* promoter reporting line 2, did not exhibit detectable levels *DIN3::LUC* expression. Since only three out of nine lines expressed *DIN3::LUC* at detectable levels and none exhibited the characteristic induction of *DIN3::LUC* following a 6h dark treatment, the effect of the mutation in the version of *DIN3::LUC* expressed by Scan Line 9 can be said to have disrupted *DIN3::LUC* expression compared to native *DIN3* expression. The Scan Line 9 mutation changes ATCCTC of the native sequence to GAATTC; no known cis-acting motifs in part or entirety were detected within the 6 base-pairs of the EcoRI substitution.



**Figure 3.5.10 Mean *DIN3::LUC* expression in 7-day-old Scan Line 9 seedlings following six-hour dark treatment.**

Mean *DIN3::LUC* expression, determined by luminometry, in 7-day-old Scan Line 9 seedlings following either 6h dark treatment. The error bars represent the standard error of the mean. Control Line 2 (RL2) was included as a comparison; this line expresses the unmodified 300bp of the *DIN3* promoter fused to luciferase. These data are not normally distributed as can be seen by the larger S.E.M. for those data with greater expression. These data were transformed by a  $\log_{10}(x+1)$  transformation and compared with the background luminescence. The asterisk (\*) indicates those data sets which were at least 2 S.E.M. from the background.

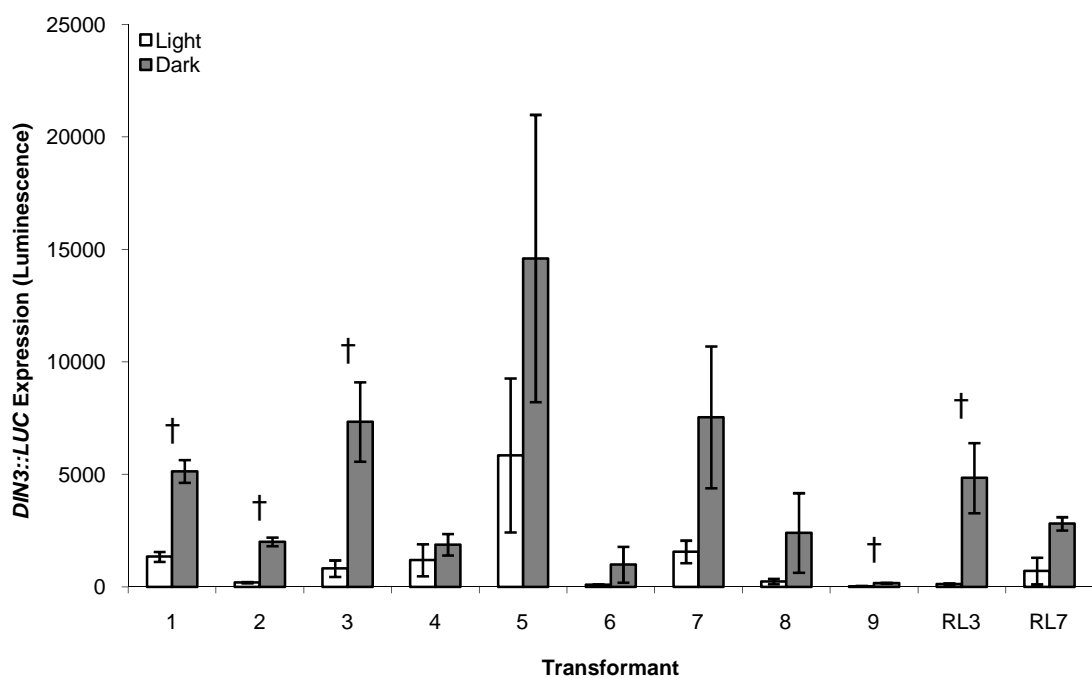
In all but lines 6 and 13, the transformant lines of Scan Line 10 exhibited *DIN3::LUC* expression which was greater following the 6h dark treatment compared with the 6h light treatment (fig. 3.5.11). However with transformant lines 3, 6, 8, 11, 13 and *DIN3* promoter reporting line 3 this difference was not significant (fig. 3.5.11). Only transformant line 8 of Scan Line 10 was *DIN3::LUC* expression undetectable following 6h dark treatment. Transformant lines 2, 9, 12 and 15 of Scan Line 10 exhibited *DIN3::LUC* expression greater than that of *DIN3* promoter reporting line 2, up to 2.3 times in the case of transformant line 15. With 10 out of 15 transformant lines demonstrating significant dark inducible *DIN3::LUC* expression patterns, the modified *DIN3::LUC* expressed by Scan Line 10 seedlings appears unaffected when compared with native *DIN3* expression. The Scan Line 10 mutation changes TCTCCG of the native sequence to GAATTC; no known cis-acting motifs in part or entirety were detected within the 6 base-pairs of the EcoRI substitution.



**Figure 3.5.11 Mean *DIN3::LUC* expression in 7-day-old Scan Line 10 seedlings following six-hour light and dark treatment**

Mean *DIN3::LUC* expression, determined by luminometry, in 7-day-old Scan Line 10 seedlings following either 6h light (white) or dark (grey) treatment. The error bars represent the standard error of the mean. *DIN3* promoter reporting line 2 and 3 (RL2/3) were included for comparison; these lines express the unmodified 300bp of the *DIN3* promoter fused to luciferase. These data are not normally distributed as can be seen by the larger S.E.M. for those data with greater expression. These data were transformed by a  $\log_{10}(x+1)$  transformation and compared with the background luminescence. All data sets (except line 8) were at least 2 S.E.M. from the background. A figure for this analysis is available in the appendix. The dagger (†) indicates those transformant lines whose light/dark *DIN3::LUC* expression was >2 S.E.M. apart.

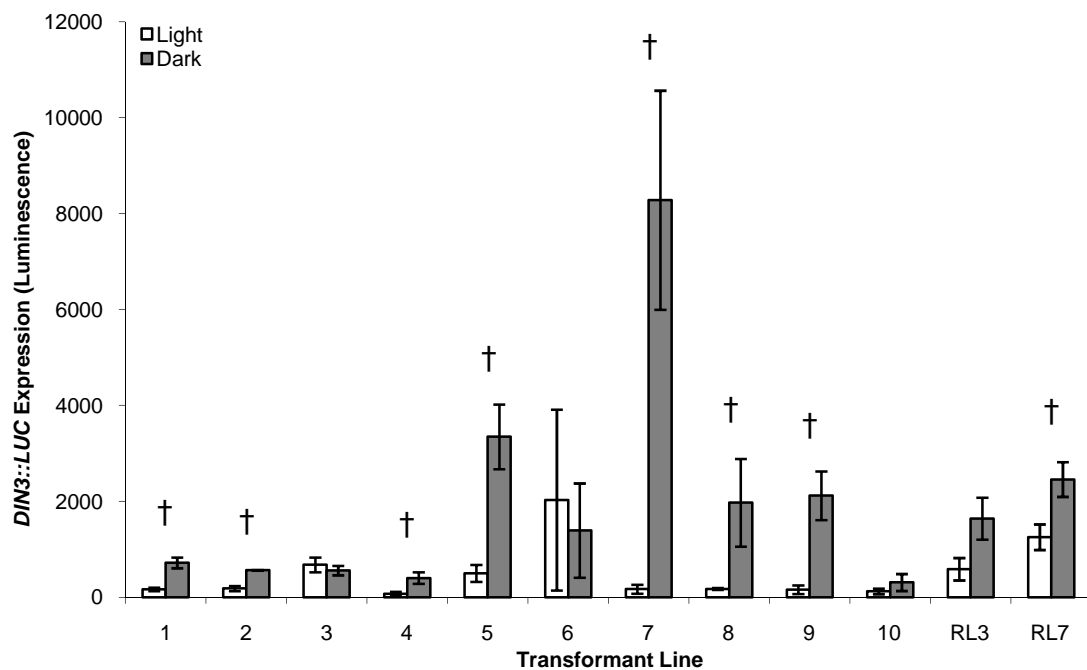
In all the transformant lines of Scan Line 11 exhibited *DIN3::LUC* expression which was greater following the 6h dark treatment compared with the 6h light treatment (fig. 3.5.12). However with transformant lines 4, 5, 6, 7, 8 and *DIN3* promoter reporting line 7 this difference was not significant (fig. 3.5.12). Only in transformant lines 6 and 8 of Scan Line 11 was *DIN3::LUC* expression undetectable following 6h dark treatment. Transformant lines 1, 3, 5 and 7 of Scan Line 11 exhibited *DIN3::LUC* expression greater than that of *DIN3* promoter reporting line 3, up to 3 times in the case of transformant line 5. With all nine transformant lines demonstrating dark induction of *DIN3::LUC* expression and four out of nine significantly, the modified *DIN3::LUC* expressed by Scan Line 11 seedlings appears unaffected when compared with native *DIN3* expression. The Scan Line 11 mutation changes CGTGAT of the native sequence to GAATTC; a GATA cis-acting motif (GATA) was detected within the 6 base-pairs of the EcoRI substitution.



**Figure 3.5.12 Mean *DIN3::LUC* expression in 7-day-old Scan Line 11 seedlings following six-hour light and dark treatment**

Mean *DIN3::LUC* expression, determined by luminometry, in 7-day-old Scan Line 11 seedlings following either 6h light (white) or dark (grey) treatment. The error bars represent the standard error of the mean. *DIN3* promoter reporting line 3 and 7 (RL3/7) were included for comparison; these lines express the unmodified 300bp of the *DIN3* promoter fused to luciferase. These data are not normally distributed as can be seen by the larger S.E.M. for those data with greater expression. These data were transformed by a  $\log_{10}(x+1)$  transformation and compared with the background luminescence. All data sets (except lines 6 & 8) were at least 2 S.E.M. from the background. A figure for this analysis is available in the appendix. The dagger (†) indicates those transformant lines whose light/dark *DIN3::LUC* expression was >2 S.E.M. apart.

In all but lines 3 and 6, the transformant lines of Scan Line 12 exhibited *DIN3::LUC* expression which was greater following the 6h dark treatment compared with the 6h light treatment (fig. 3.5.13). However with transformant lines 3, 6, 10 and *DIN3* promoter reporting line 3 this difference was not significant (fig. 3.5.13). Only in transformant lines 6 and 10 of Scan Line 12 was *DIN3::LUC* expression undetectable following 6h dark treatment. Transformant lines 5 and 7 of Scan Line 12 exhibited *DIN3::LUC* expression greater than that of *DIN3* promoter reporting lines 7, up to 3.4 times in the case of transformant line 7. With eight out of 10 transformant lines demonstrating dark induction of *DIN3::LUC* expression and seven significantly, the modified *DIN3::LUC* expressed by Scan Line 12 seedlings appears unaffected when compared with native *DIN3* expression. The Scan Line 12 mutation changes ATATTG of the native sequence to GAATTC; a CCAATBOX1 cis-acting motif (CCAAT) was detected within the 6 base-pairs of the EcoRI substitution.



**Figure 3.5.13 Mean *DIN3::LUC* expression in 7-day-old Scan Line 12 seedlings following six-hour light and dark treatment**

Mean *DIN3::LUC* expression, determined by luminometry, in 7-day-old Scan Line 12 seedlings following either 6h light (white) or dark (grey) treatment. The error bars represent the standard error of the mean. *DIN3* promoter reporting lines 3 and 7 (RL3/7) were included for comparison; these lines express the unmodified 300bp of the *DIN3* promoter fused to luciferase. These data are not normally distributed as can be seen by the larger S.E.M. for those data with greater expression. These data were transformed by a  $\log_{10}(x+1)$  transformation and compared with the background luminescence. All data sets (except lines 6 & 10) were at least 2 S.E.M. from the background. A figure for this analysis is available in the appendix. The dagger (†) indicates those transformant lines whose light/dark *DIN3::LUC* expression was >2 S.E.M. apart.

### 3.6 Linker Scan Analysis of dark and cold *DIN3::LUC* expression

The luminometry method of following gene expression is a proxy for analysing the transient level of the gene of interest and requires the reporter gene (*luc<sup>INT</sup>*) (Mankin et al., 1997) to be successfully transcribed and translated to luciferase. Using the luminometry method the effect of the linker-scan mutations on the dark inducible expression of *DIN3::LUC* was investigated. Herein, the effect of those same mutations on the dark induction and the effect of low temperature on *DIN3::LUC* expression will be considered. Various endogenous factors can influence and affect translation; additionally environmental factors too can have a great effect. The cold treatment used in these experiments, is just such an environmental factor that affects translation such that, the luminometry method is rendered useless for analysing gene expression after cold treatment. This problem can be overcome by analysis of gene expression directly from the RNA present in the seedlings at the end of the treatment in a process called quantitative Real-Time PCR (qRT-PCR). However using qRT-PCR to analysis the expression of the *DIN3::LUC* does not take account of 3'UTR of *DIN3* which is missing having been replaced by the luciferase (*luc<sup>INT</sup>*) gene. This issue was addressed by the first experiment (fig. 3.6.1) described in this section. qRT-PCR provides quantitative assay of gene expression, although all qRT-PCR analyses in this work use a relative, rather than absolute, quantitative method. qRT-PCR also has the potential to be more sensitive than luminometry or methods relying on the successful translation of the reporter gene for the reasons mentioned earlier.

The approach taken in this section was similar to that of the previous using luminometry. Seedlings of the various scan lines were grown to 7 days old and then underwent treatments consisting of either three hours of light or darkness at 20°C or darkness at 5°C. About 25 seedlings were then harvested and their RNA extracted and cDNA synthesised, whereupon it was analysed by qRT-PCR for the expression levels of *DIN3* and *DIN3::LUC*. This section will consider the effect that the promoter modifications had on low temperature regulation of *DIN3::LUC* expression.

For both *DIN3* and *DIN3::LUC*, the expression is represented relative to that of the light sample. The gene expression shown in this and following analyses is therefore not an absolute measure of gene transcripts in a given sample. This first experiment was carried out on 7-day-old *DIN3* promoter reporting line 2 seedlings expressing the unmodified *DIN3::LUC* gene. Following every treatment, the expression of *DIN3::LUC* is compared against the expression of *DIN3* which acts as an endogenous control. It should be noted that a single sample of seedlings was taken from each environmental treatment and this one sample went on to produce the expression measurement of each gene, hence the expression of the two genes can be directly compared.

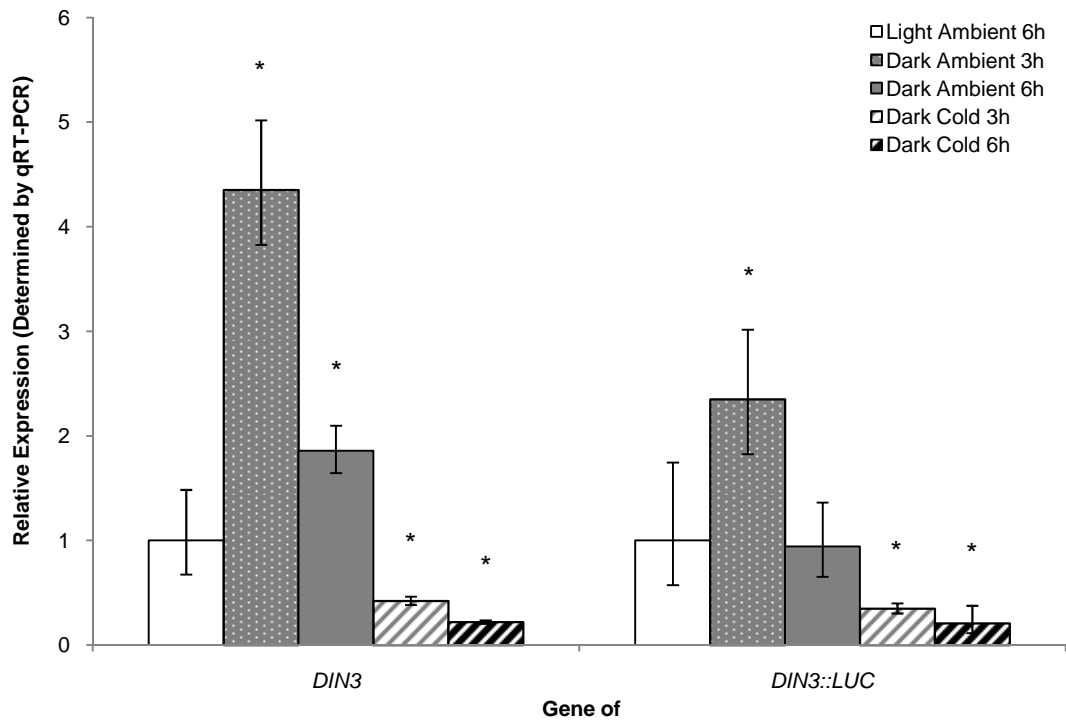
Starting with *DIN3* expression, seedlings which underwent ambient (20°C) dark treatments of three hours or six hours had greater *DIN3* expression than those which remained in the light as expected. The increase in *DIN3* expression was greatest after three hours (4.3 times relative to the light treated seedlings) and declined after six hours (1.9 times relative to the light treatment) (fig. 3.6.1). However without a three-hour time point for the light treated seedlings this comparison cannot be made directly and the three-hour peak may be artefactual. When dark treatment was carried out under cold conditions (5°C), this induction of *DIN3* expression was not observed. In fact amongst those seedlings treated to three and six hours of darkness at 5°C had 0.4 and 0.2 times of *DIN3* expression respectively relative to light treated expression (fig. 3.6.1).

Considering now *DIN3::LUC* expression, it is immediately evident that the pattern of luciferase expression mirrored that of the native *DIN3* expression (fig. 3.6.1). This is a crucial finding since it demonstrates that the *DIN3::LUC* expression changes in response to a change in environmental conditions in the same manner as the native *DIN3*. This proves that despite lacking the 3' UTR, the 300bp portion of the *DIN3* promoter fused to *luc<sup>INT</sup>* is sufficient for normal regulation of *DIN3* in response to the environmental conditions used in these experiments. It also demonstrates that *DIN3::LUC* is a reliable reporter of the effects of the linker-scanning mutations in the versions *DIN3::LUC* expressed by the scan lines and hence the effect of the disruption of various motifs found at the loci of the mutations (fig. 3.5.1). The magnitude of the changes in *DIN3::LUC* expression across the treatments was less than that of native *DIN3*. Seedlings given either three or six hours of darkness at 20°C had *DIN3::LUC* expression 2.4 and 0.9 times of that of the light treated sample respectively. Seedlings

treated with three or six hours of darkness at 5°C had *DIN3::LUC* expression levels 0.3 and 0.2 times that of the light treated seedlings (fig. 3.6.1).

In summary, *DIN3* expression increases following a period of darkness at 20°C peaking sometime around three hours and declining after that. Without a three-hour time point for the light treated seedlings against which to compare it this peak may be artefactual. This induction of *DIN3* expression is not seen when the dark treatment is combined with exposure to cold conditions (5°C); *DIN3* expression declines relative to basal expression after three hours, but even further after six hours of darkness at 5°C (fig. 3.6.1).

*DIN3::LUC* expression mirrors this pattern almost precisely, with an induction of its expression following a period of darkness at 20°C. This treatment appears most potent around three hours; its effect declines beyond this so that after six hours of darkness at 20°C, *DIN3::LUC* expression levels are similar to those seen in the light treated seedlings. The same inhibition of the dark induction is seen with *DIN3::LUC* expression when seedlings are exposed to darkness and chilling conditions; expression levels are fractions of the basal expression and the effect seems to increase with time (fig. 3.6.1). It is worth stressing that this experiment has demonstrated that, since *DIN3::LUC* exhibited the same expression patterns as *DIN3*, it clearly possesses a sufficient portion of the *DIN3* promoter to study cold regulation via linker-scanning mutagenesis.



**Figure 3.6.1 *DIN3* and *DIN3::LUC* expression in 7-day-old *DIN3* promoter reporting line 2 seedlings following six hour light ambient and three and six hour dark ambient and dark cold treatments**

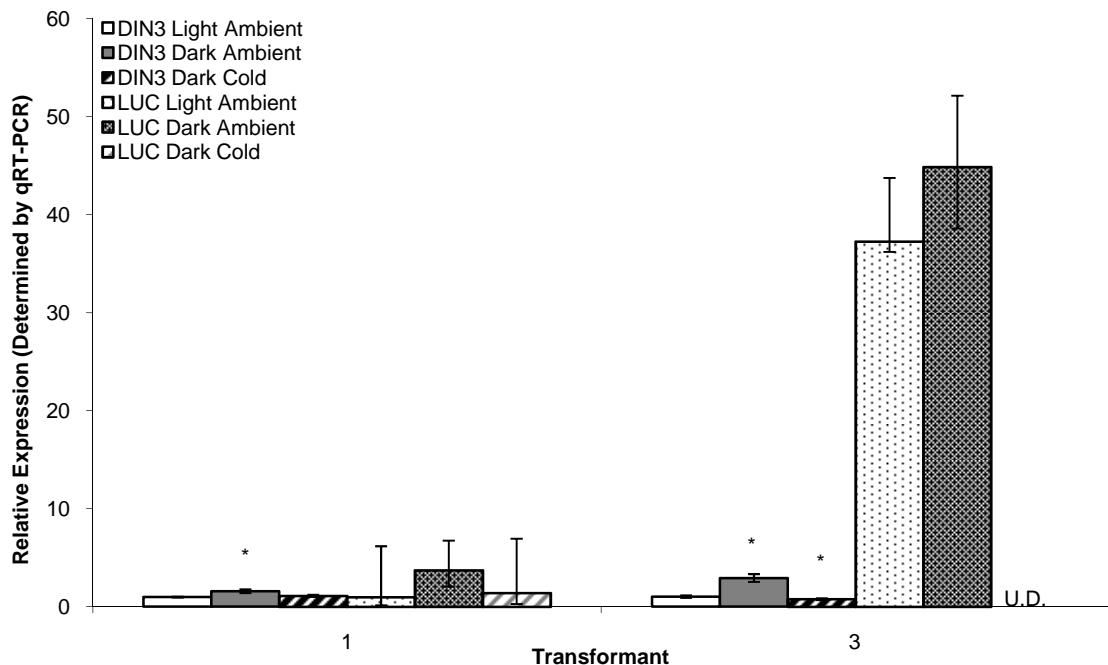
Relative *DIN3* and *DIN3::LUC* expression levels in 7-day-old *DIN3* promoter reporting line 2 (standardised against *TUB4* expression) seedlings after 6h light ambient (20°C) (white), 3h dark ambient (grey dots), 6h dark ambient (solid grey), 3h dark cold (5°C) (grey stripes) or 6h dark cold (black stripes) treatments. Expression levels are relative to the light sample. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; *df*=4.

In all subsequent experiments carried out on the scan lines, three hour treatments were employed since this produced the greatest and therefore most easily detectable change in expression levels. The results of first qRT-PCR analyses informed the decisions made as to which transformant lines from each scan line would be used in the final cold temperature regulation experiments, the results of which are presented in the figures of this section. A link between the *DIN3::LUC* expression measured via luminometry and the quality of qRT-PCR results was formed so that transformant lines exhibiting approximately median level of *DIN3::LUC* expression for the scan line were chosen for the experiments. As far as possible three representative transformant lines were chosen, but in some cases the *DIN3::LUC* expression levels were so low that this was not possible. *DIN3* expression was also measured for all the scan lines, to act as an endogenous control and to provide confirmation that the experimental treatments were carried out correctly eliciting the characteristic native response.

In both transformants 1 and 3 of Scan Line 1 the pattern of *DIN3* expression across the three treatments matched that of response of *DIN3* promoter reporting line 2 (figs. 3.6.2 & 3.6.1). However the magnitude of the response was very small in both cases. Three hours of darkness at 20°C elicited *DIN3* expression only 1.6 and 2.9 times over basal expression in transformant lines 1 and 3 respectively. The three hour dark treatment at 5°C, as anticipated, inhibited any dark induction so that *DIN3* expression was 1.1 and 0.8 times that of basal expression (fig. 3.6.2)

Considering now the *DIN3::LUC* expression following the three hour treatments, transformant line 1 exhibited the characteristic pattern and as with *DIN3*, the magnitude of the response was less than anticipated: 3.6 and 1.4 times basal expression following three hour dark treatments at 20°C and 5°C respectively (fig. 3.6.2). *DIN3::LUC* expression in transformant line 3 also exhibited the characteristic pattern over the treatments. However the overall magnitude of expression was much greater than transformant line 1, to which its expression is relative. The light associated *DIN3::LUC* expression of transformant line 3 was 37.3 times that of line 1. Three hours of darkness at 20°C induced *DIN3::LUC* expression which was 44.9 times that of the basal expression of line 1, although only 1.2 times that of line 3. *DIN3::LUC* expression following three hours of darkness at 5°C provided undetectable (figs. 3.6.2 & 3.6.1).

Overall the transformant lines 1 and 3 of Scan Line 1 exhibited the characteristic dark induction of *DIN3::LUC* following three hours of darkness at 20°C and an inhibition of this increase in expression over basal levels following three hours of darkness at 5°C for *DIN3* and *DIN3::LUC* genes. The light sample of transformant 3 exhibited exceptionally high levels of *DIN3::LUC* expression when compared with transformant 1 or its own *DIN3* expression. Despite this the *DIN3::LUC* expression pattern remained the same as *DIN3*. *DIN3::LUC* expression across the treatments appears unaffected by Scan Line 1 mutation, which changes GTGATG of the native sequence to GAATTC; no known cis-acting motifs in part or entirety were detected within the 6 base-pairs of the EcoRI substitution.

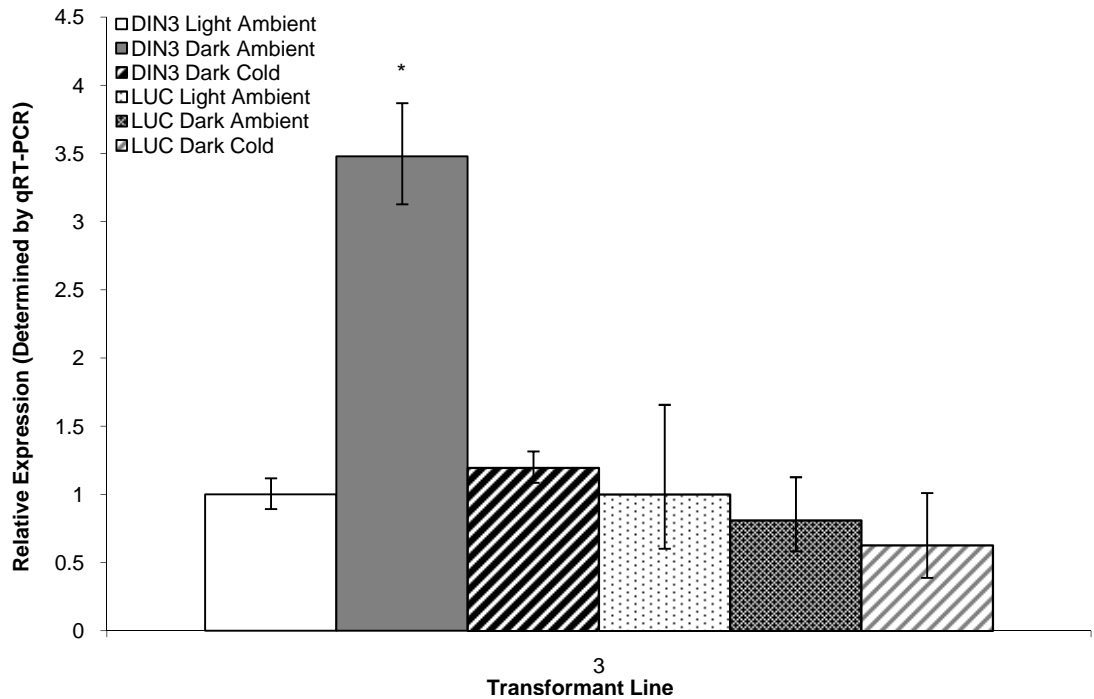


**Figure 3.6.2 *DIN3* and *DIN3::LUC* expression in 7-day-old Scan Line 1 seedlings following three hour light ambient, dark ambient and dark cold treatments**

Figure 3.6.2 Relative *DIN3* (solid colours) and *DIN3::LUC* (textured) expression levels in 7-day-old Scan Line 1 (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. Expression levels are relative to the light sample of transformant line 1. U.D. = Expression Undetermined i.e. so low as beyond the limits of detectability. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

In the case of Scan Line 2, only one transformant line had both suitable seedling growth and sufficient *DIN3::LUC* expression, as determined by luminometry, to be viable for analysis by qRT-PCR to produce meaningful data. The *DIN3* expression across the treatments for Scan Line 2 matched the expected pattern with a clear dark induction with 3.5 times *DIN3* expression in seedlings treated with three hours of darkness at 20°C compared with those remaining in the light. This induction was inhibited in those seedlings which experienced three hours of darkness at 5°C whose *DIN3* expression was 1.2 times that of basal expression (fig. 3.6.3). In those same seedlings, *DIN3::LUC* expression did not mirror that of *DIN3*. Three hours of darkness at 20°C failed to induce *DIN3::LUC* expression in Scan Line 2 with 0.8 times that of basal expression. After three of darkness at 5°C the *DIN3::LUC* expression was even lower: 0.6 of basal expression (fig. 3.6.3).

Scan Line 2 exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treatment was combined with 5°C. However this pattern was not seen with the *DIN3::LUC* expression. No dark induction of *DIN3::LUC* was triggered by the three hours of darkness at 20°C. Without a dark-ambient induction against which to compare it, dark-cold inhibition of *DIN3::LUC* is meaningless. The Scan Line 2 mutation changes TGTACA of the native sequence to GAATTC, no known cis-acting motifs no known cis-acting motifs in part or entirety were detected within the 6 base-pairs of the EcoRI substitution. The effect of the mutation in the version of *DIN3::LUC* expressed by Scan Line 2 can be said to have disrupted *DIN3::LUC* expression compared to native *DIN3* expression.



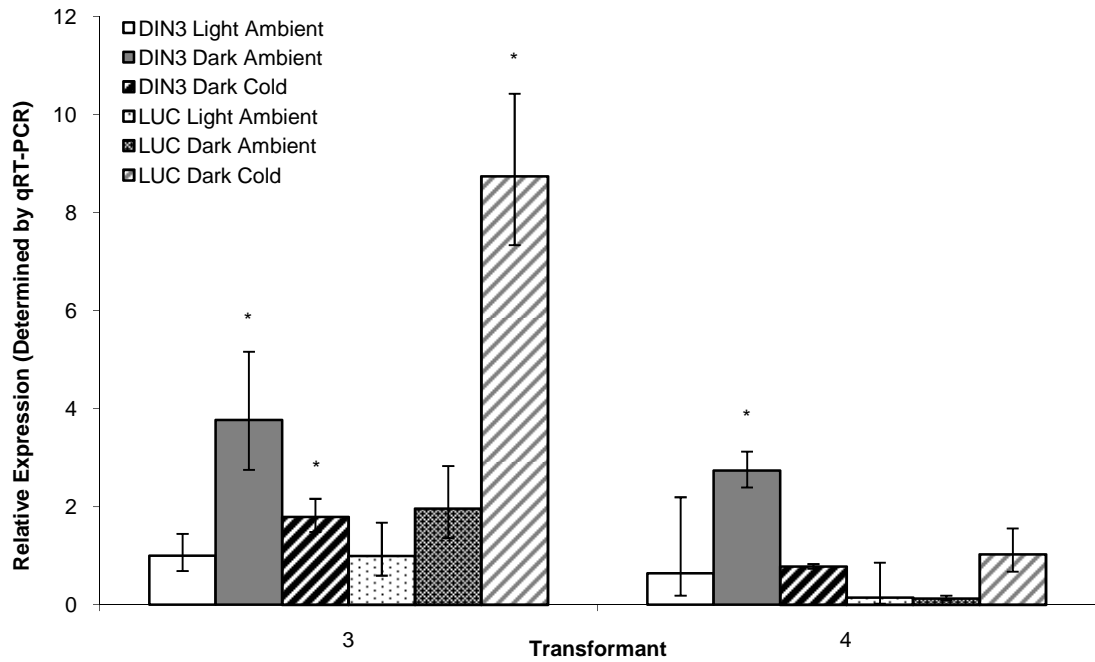
**Figure 3.6.3 *DIN3* and *DIN3::LUC* expression in 7-day-old Scan Line 2 seedlings following three hour light ambient, dark ambient and dark cold treatments**

Relative *DIN3* (solid colours) and *DIN3::LUC* (textured) expression levels in 7-day-old Scan Line 2 (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. Expression levels are relative to the light sample. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; *df*=4.

The *DIN3* expression across the treatments for Scan Line 3 matched the expected pattern with a clear dark induction with 3.5 and 2.4 times *DIN3* expression in seedlings of transformant lines 3 and 4 respectively, which were treated with three hours of darkness at 20°C, compared with transformant line 3 seedlings remaining in the light. This induction was inhibited in those seedlings which experienced three hours of darkness at 5°C whose *DIN3* expression was 1.7 and 0.7 times that of basal expression in lines 3 and 4 respectively (fig. 3.6.4).

In those same seedlings, *DIN3::LUC* expression did not mirror that of *DIN3*. Three hours of darkness at 20°C induced *DIN3::LUC* expression in transformant line 3, although perhaps weakly, with 1.8. Dark-induction was absent in transformant line 4: 0.8 times that of basal expression after three hours of darkness at 20°C. The dark-cold inhibition of *DIN3::LUC* was absent in transformant line 3; after three of darkness at 5°C the *DIN3::LUC* expression was elevated: 7.7 of basal expression. With the presence of dark-induction of *DIN3::LUC* expression in the seedlings of transformant line 4 the dark-cold inhibition of *DIN3::LUC* cannot be determined (fig. 3.6.4).

Scan Line 3 exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treatment was combined with 5°C. However this pattern was not seen with the *DIN3::LUC* expression. Weak dark-induction of *DIN3::LUC* was triggered by the three hours of darkness at 20°C in transformant line 3 but not in line 4. The characteristic dark-cold inhibition of *DIN3::LUC* was entirely absent in transformant line 3 and without dark-induction in transformant line 4, the maintenance of dark-cold inhibition is meaningless. The Scan Line 3 mutation changes CATGAG of the native sequence to GAATTC, a MYBST1 cis-acting motif (CGATA) was detected within the 6 base-pairs of the EcoRI substitution. The effect of the mutation in the version of *DIN3::LUC* expressed by Scan Line 3 can be said to have disrupted *DIN3::LUC* expression compared to native *DIN3* expression.



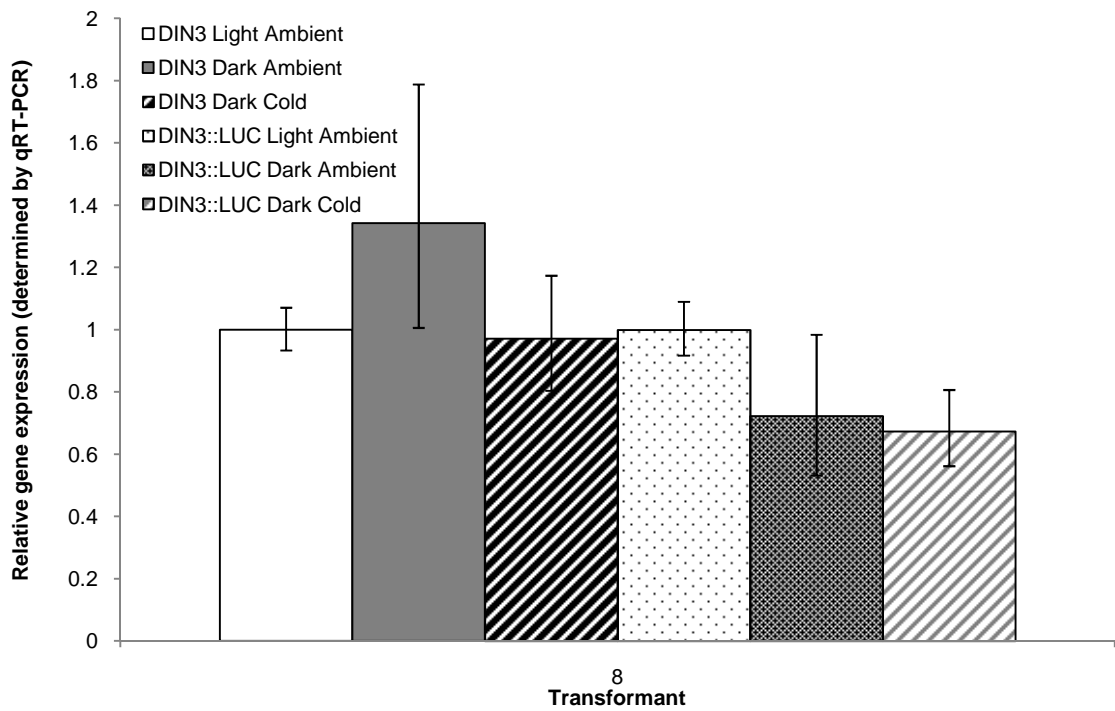
**Figure 3.6.4 *DIN3* and *DIN3::LUC* expression in 7-day-old Scan Line 3 seedlings following three hour light ambient, dark ambient and dark cold treatments**

Relative *DIN3* (solid colours) and *DIN3::LUC* (textured) expression levels in 7-day-old Scan Line 3 (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. Expression levels are relative to the light sample of transformant line 3. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

It should be noted that the data presented in figure 3.6.5 are the products of two independent experiments; a second was carried out to reduce the variance in the data. Additionally, only one transformant line of Scan Line 4 was found to express *DIN3::LUC* at a level sufficient for qRT-PCR analysis. The *DIN3* expression across the treatments for Scan Line 4 matched the expected pattern with a weak dark-induction 1.3 times *DIN3* expression in seedlings treated with three hours of darkness at 20°C compared with those remaining in the light. This induction was inhibited in those seedlings which experienced three hours of darkness at 5°C whose *DIN3* expression was 0.97 times that of basal expression (fig. 3.6.5).

In those same seedlings, *DIN3::LUC* expression did not mirror that of *DIN3*. Three hours of darkness at 20°C failed to induce *DIN3::LUC* expression in Scan Line 4, with 0.72 times that of basal expression. Without dark-induction, nothing conclusive can be said of the dark-cold inhibition of *DIN3::LUC* in Scan Line 4; after three of darkness at 5°C the *DIN3::LUC* expression 0.67 times that of basal expression (fig. 3.6.5).

Scan Line 4 exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treatment was combined with 5°C. However this pattern was not seen with the *DIN3::LUC* expression. No dark-induction of *DIN3::LUC* was seen after three hours of darkness at 20°C in Scan Line 4. The without dark-induction for comparison, the presence of dark-cold inhibition of *DIN3::LUC* cannot be determined. The Scan Line 4 mutation changes AGGATA of the native sequence to GAATTC, the MYBST1 (CGATA), GATABOX (GATA) and DPBFCOREDCDC (CGCGTGT) cis-acting motifs were detected in part or entirety were detected within the 6 base-pairs of the EcoRI substitution. The effect of the mutation in the version of *DIN3::LUC* expressed by Scan Line 4 can be said to have disrupted *DIN3::LUC* expression compared to native *DIN3* expression.



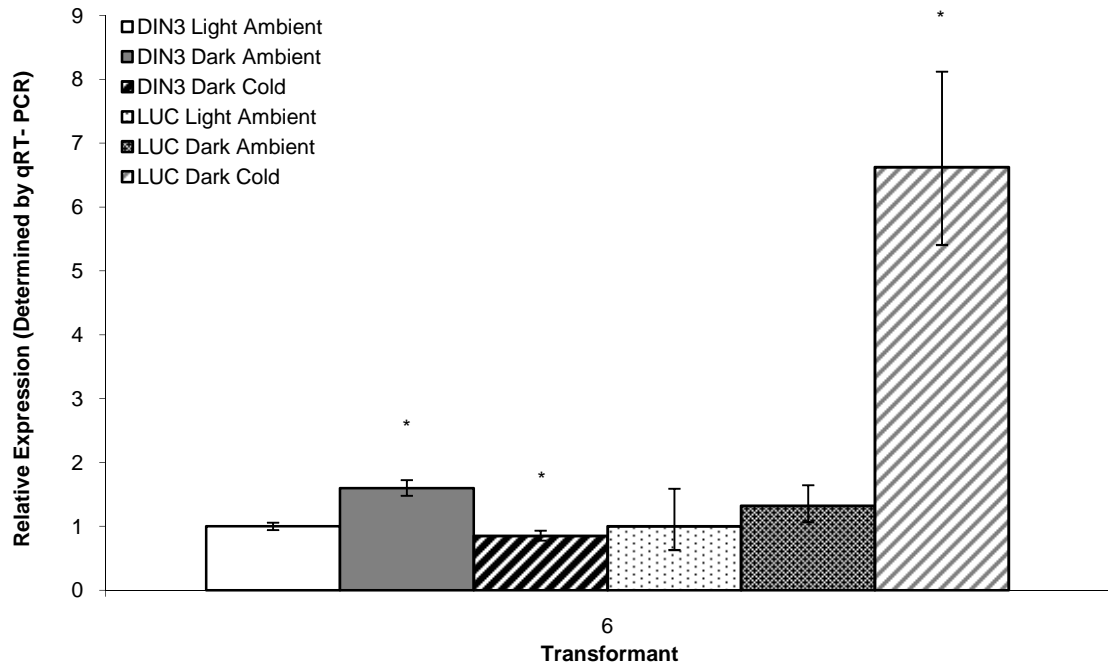
**Figure 3.6.5** *DIN3* and *DIN3::LUC* expression in 7-day-old Scan Line 4 seedlings following three hour light ambient, dark ambient and dark cold treatments

Relative *DIN3* (solid colours) and *DIN3::LUC* (textured) expression levels in 7-day-old Scan Line 4 (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. Expression levels are relative to the light sample. Error bars represent the Student's *t*-distribution maxima and minima; df=7.

Only one transformant line of Scan Line 6 was found to express *DIN3::LUC* at a level sufficient for qRT-PCR analysis. The *DIN3* expression across the treatments for Scan Line 6 matched the expected pattern with a weak dark-induction 1.6 times *DIN3* expression in seedlings treated with three hours of darkness at 20°C compared with those remaining in the light. This induction was inhibited in those seedlings which experienced three hours of darkness at 5°C whose *DIN3* expression was 0.9 times that of basal expression (fig. 3.6.6).

In those same seedlings, *DIN3::LUC* expression did not mirror that of *DIN3*. Three hours of darkness at 20°C induced *DIN3::LUC* expression in Scan Line 6, although weakly, with 1.3 times that of basal expression. The dark-cold inhibition of *DIN3::LUC* was absent in Scan Line 6; after three hours of darkness at 5°C the *DIN3::LUC* expression was elevated: 6.6 of basal expression (fig. 3.6.5).

Scan Line 6 exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treatment was combined with 5°C. However this pattern was not seen with the *DIN3::LUC* expression. Weak dark-induction of *DIN3::LUC* was triggered by the three hours of darkness at 20°C in Scan Line 6. The characteristic dark-cold inhibition of *DIN3::LUC* was entirely absent with elevated *DIN3::LUC* expression following the dark-cold treatment. The Scan Line 6 mutation changes CGTGTA of the native sequence to GAATTC; ABRERATCAL (ACGCGTG), CGCGBOXAT (ACGCGT) cis-acting motifs in part or entirety were detected within the 6 base-pairs of the EcoRI substitution. The effect of the mutation in the version of *DIN3::LUC* expressed by Scan Line 6 can be said to have disrupted *DIN3::LUC* expression compared to native *DIN3* expression.



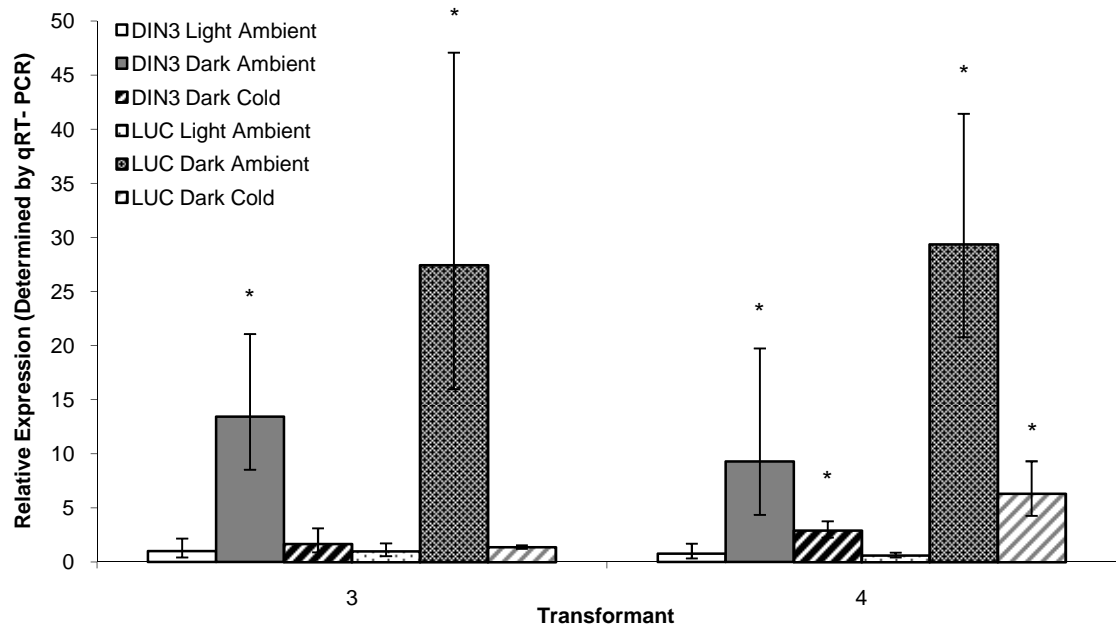
**Figure 3.6.6 *DIN3* and *DIN3::LUC* expression in 7-day-old Scan Line 6 seedlings following three hour light ambient, dark ambient and dark cold treatments**

Relative *DIN3* (solid colours) and *DIN3::LUC* (textured) expression levels in 7-day-old Scan Line 6 (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. Expression levels are relative to the light sample. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; *df*=4.

The *DIN3* expression across the treatments for Scan Line 7 matched the expected pattern, with a strong dark-induction: 13.4 and 9.3 times *DIN3* expression in seedlings of transformant lines 3 and 4 respectively, when treated with three hours of darkness at 20°C compared with the transformant line 3 light sample. This induction was inhibited in seedlings of transformant lines 4 and 5 which experienced three hours of darkness at 5°C whose *DIN3* expression was 1.7 and 2.9 times that of basal expression, respectively. In all cases the differences quoted were significantly different from the transformant line 3 light sample (fig. 3.6.7).

In those same seedlings, *DIN3::LUC* expression mirrored that of *DIN3*. Three hours of darkness at 20°C elicited an induction in *DIN3::LUC* expression by 27.5 and 29.4 times that of the basal expression in transformant lines 3 and 4 respectively. Following three hours in darkness at 5°C *DIN3::LUC* induction was inhibited, with expression 1.4 and 6.3 times that of the light sample in transformant lines 3 and 4 respectively (fig. 3.6.7).

Scan Line 7 exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treatment was combined with 5°C. This pattern was replicated by the *DIN3::LUC* expression, with dark induction of *DIN3::LUC* by the three hours of darkness at 20°C and its inhibition by the cold treatment. The Scan Line 7 mutation changes TACCGT of the native sequence to GAATTC; a MYB2CONSENSUSAT cis-acting motif (CCGTTG) in part or entirety were detected within the 6 base-pairs of the EcoRI substitution. The mutation in the version of *DIN3::LUC* expressed by the Scan Line 7 seedlings did not disrupt or eliminate the characteristic dark induction seen in the native *DIN3* expression.



**Figure 3.6.7 *DIN3* and *DIN3::LUC* expression in 7-day-old Scan Line 7 seedlings following three hour light ambient, dark ambient and dark cold treatments**

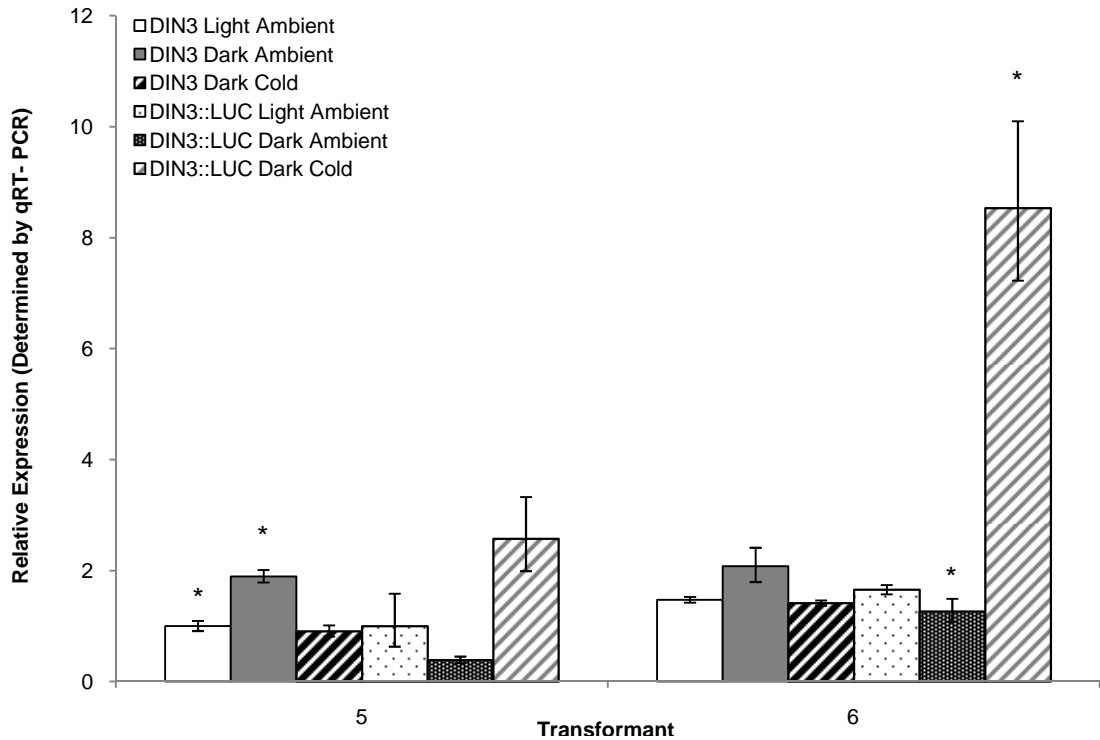
Relative *DIN3* (solid colour) and *DIN3::LUC* (textured) expression levels in 7-day-old Scan Line 7 (standardised against *TUB4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. Expression levels are relative to the light sample of transformant line 3. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; *df*=4.

It should be noted that the data presented in figure 3.6.8 are the products of two independent experiments; a second was carried out to reduce the variance in the data. The *DIN3* expression across the treatments for Scan Line 8 matched the expected pattern with clear dark-induction with 1.9 and 2.1 times *DIN3* expression in seedlings of transformant lines 5 and 6 respectively, when treated with three hours of darkness at 20°C compared with transformant line 5 light sample. This induction was inhibited in seedlings of transformant lines 5 and 6 which experienced three hours of darkness at 5°C whose *DIN3* expression was 0.9 and 1.4 times that of basal expression respectively (fig. 3.6.8).

In those same seedlings, *DIN3::LUC* expression did not mirror that of *DIN3*. Three hours of darkness at 20°C failed to induce *DIN3::LUC* expression in transformant line 5: 0.4 times basal. Neither was dark-induction seen in line 6: 1.3 times the basal expression, but lower than its own light sample. Without dark-induction of *DIN3::LUC* expression commentary on cold-inhibition is meaningless; however *DIN3::LUC* following the three hours of darkness at 5°C 2.6 and 8.5 times basal expression respectively (fig. 3.6.8).

Scan Line 8 exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treatment was combined with 5°C. However this pattern was not seen with the *DIN3::LUC* expression. The three hours of darkness at 20°C failed to induce either transformant line 5 or 6 of Scan Line 8. With no dark-induction of *DIN3::LUC*, dark-cold inhibition of *DIN3::LUC* in lines 5 and 6 cannot be determined; dark-cold expression of *DIN3::LUC* expression was however considerably above either their light or dark 20°C samples. The Scan Line 8 mutation changes GTTGAT of the native sequence to GAATTC; a MYB2CONSENSUSAT cis-acting motif (CCGTTG) was detected within the 6 base-pairs of the EcoRI substitution.

The effect of the mutation in the version of *DIN3::LUC* expressed by Scan Line 8 can be said to have disrupted *DIN3::LUC* expression compared to native *DIN3* expression.



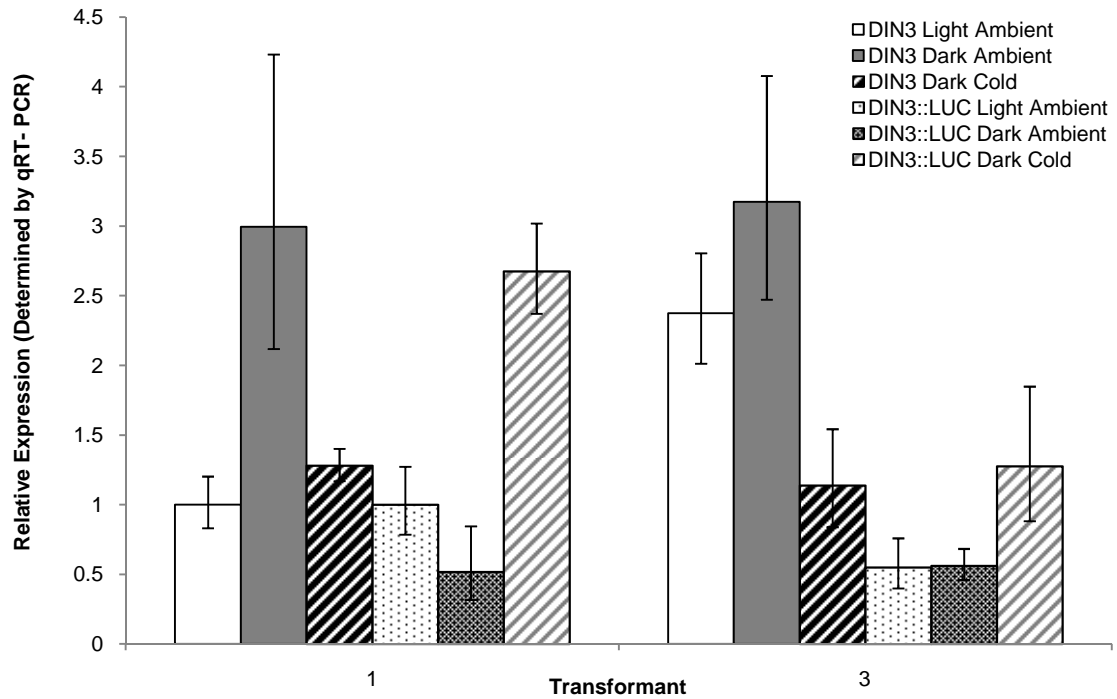
**Figure 3.6.8 *DIN3* and *DIN3::LUC* Expression in 7-day-old Scan Line 8 seedlings following three hour light ambient, dark ambient and dark cold treatments**

Relative *DIN3* (solid colour) and *DIN3::LUC* (textured) expression levels in 7-day-old Scan Line 8 (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. Expression levels are relative to the light sample of transformant line 5. U.D. = Expression Undetermined i.e. so low as beyond the limits of detectability. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; df=7.

It should be noted that the data presented in figure 3.6.9 are the products of two independent experiments; a second was carried out to reduce the variance in the data. The *DIN3* expression across the treatments for Scan Line 9 matched the expected pattern with clear dark-induction with 3 and 3.2 times *DIN3* expression in seedlings of transformant lines 1 and 3 respectively, when treated with three hours of darkness at 20°C compared with transformant line 1 light sample. This induction was inhibited in seedlings of transformant lines 1 and 3 which experienced three hours of darkness at 5°C whose *DIN3* expression was 1.3 and 1.1 times that of basal expression (fig. 3.6.9).

In those same seedlings, *DIN3::LUC* expression did not mirror that of *DIN3*. Three hours of darkness at 20°C failed to induce *DIN3::LUC* expression in any of the transformant lines; line 1 had 0.5 and line 3 0.6 times basal. Without dark-induction of *DIN3::LUC* expression it is meaningless to considering cold-inhibition of *DIN3::LUC* in lines 1 and 3; *DIN3::LUC* following the three hours of darkness at 5°C was 2.7 and 1.3 times basal expression respectively (fig. 3.6.9).

Scan Line 9 exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treatment was combined with 5°C. However this pattern was not seen with the *DIN3::LUC* expression. The three hours of darkness at 20°C failed to induce either transformant line 1 or 3 of Scan Line 9. The lack of dark-induction of *DIN3::LUC* means that nothing conclusive can be said about the cold-inhibition. The Scan Line 9 mutation changes ATCCTC of the native sequence to GAATTC; no known cis-acting motifs in part or entirety were detected within the 6 base-pairs of the EcoRI substitution. The effect of the mutation in the version of *DIN3::LUC* expressed by Scan Line 9 can be said to have disrupted *DIN3::LUC* expression compared to native *DIN3* expression.



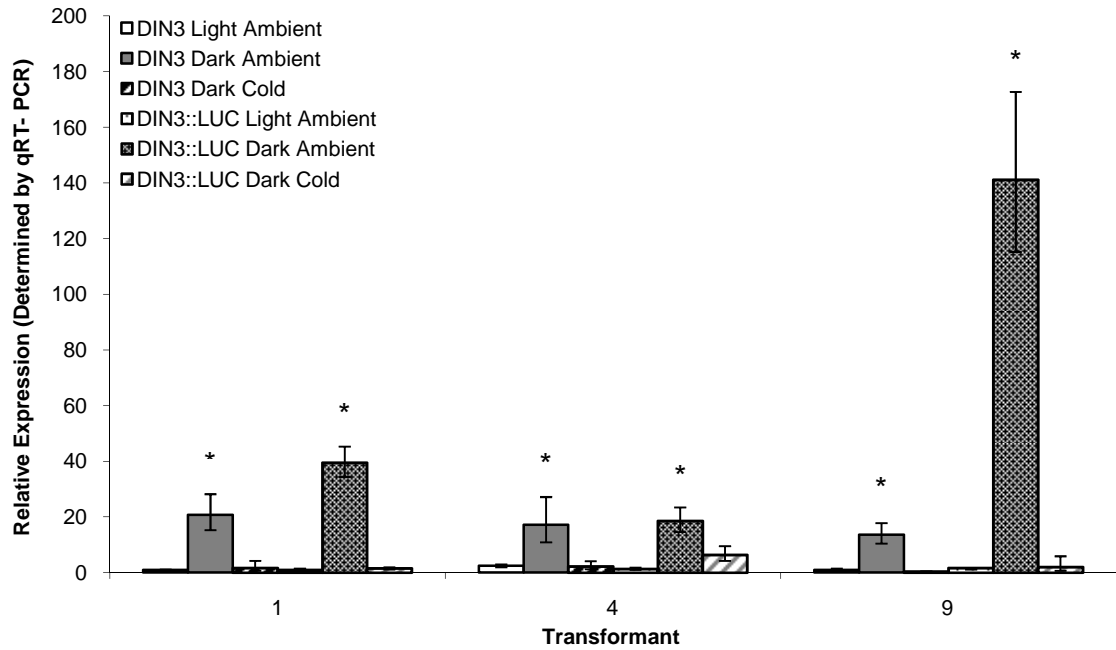
**Figure 3.6.9 *DIN3* and *DIN3::LUC* expression in 7-day-old Scan Line 9 seedlings following three hour light ambient, dark ambient and dark cold treatments**

Figure 3.6.9 Relative *DIN3* (solid colour) and *DIN3::LUC* (textured) expression levels in 7-day-old Scan Line 9 (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. Expression levels are relative to the light sample of transformant line 1. Error bars represent the Student's *t*-distribution maxima and minima; df=7.

The *DIN3* expression across the treatments for Scan Line 10 matched the expected pattern, with strong induction in lines 1, 4 and 9: 20.1, 17.2 and 13.5 times *DIN3* expression respectively, when treated with three hours of darkness at 20°C, compared with transformant line 1 light-sample. This induction was inhibited in seedlings of all lines, which experienced three hours of darkness at 5°C whose *DIN3* expression was 1.7, 1.3 and 0.3 times that of basal expression. All dark-induced *DIN3* expression was significantly different from that of the transformant 1 light sample (fig. 3.6.10).

In those same seedlings, *DIN3::LUC* expression mirrored that of *DIN3* but at much greater expression level. Three hours of darkness at 20°C induced *DIN3::LUC* expression in transformant lines 1, 4 and 9: 39.5, 18.5 and 141 times the basal expression respectively. The dark-cold inhibition of *DIN3::LUC* expression was seen in all lines, *DIN3::LUC* expression following the three hours of darkness at 5°C was 1.5, 6.5 and 2 times basal expression respectively. All dark-induced *DIN3::LUC* expression was significantly different from that of the transformant 1 light sample (fig. 3.6.10).

Scan Line 10 exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treatment was combined with 5°C. This pattern was replicated in the *DIN3::LUC* expression but with the dark-induced *DIN3::LUC* expression at several times the *DIN3* expression level. The Scan Line 10 mutation changes TCTCCG of the native sequence to GAATTC; no known cis-acting motifs in part or entirety were detected within the 6 base-pairs of the EcoRI substitution. The mutation in the version of *DIN3::LUC* expressed by the Scan Line 10 seedlings did not disrupt or eliminate the characteristic dark induction seen in the native *DIN3* expression.



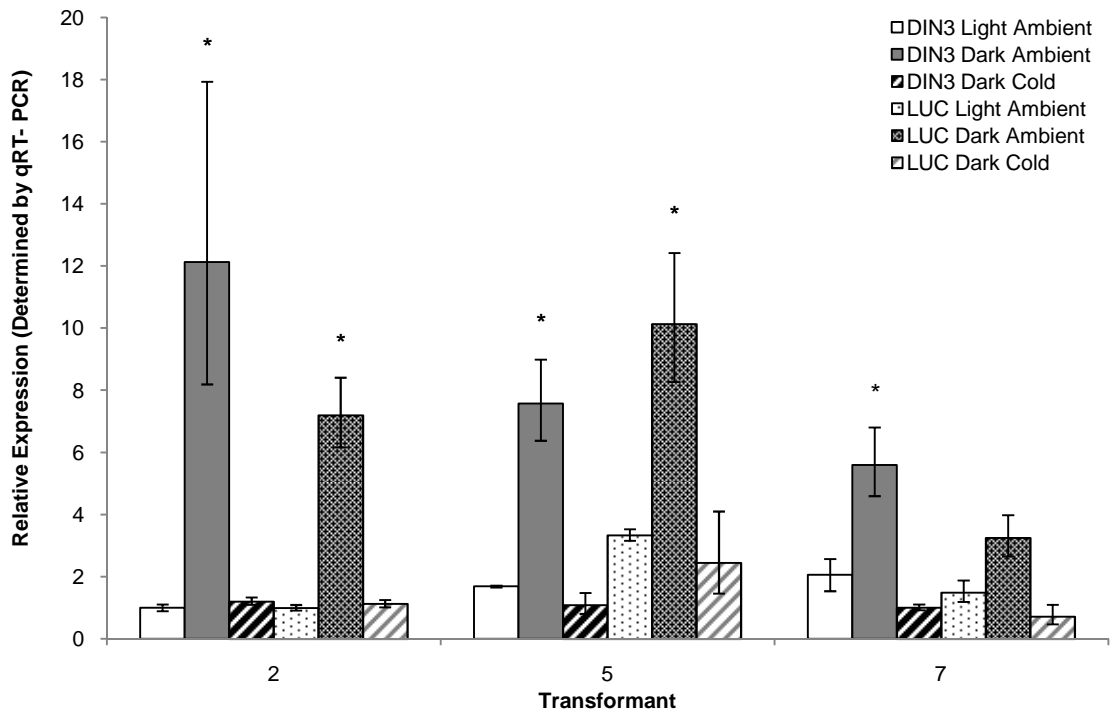
**Figure 3.6.10 *DIN3* and *DIN3::LUC* Expression in 7-day-old Scan Line 10 seedlings following three hour light ambient, dark ambient and dark cold treatments**

Figure 3.6.10 Relative *DIN3* (solid colour) and *DIN3::LUC* (textured) expression levels in 7-day-old Scan Line 10 (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. Expression levels are relative to the light sample of transformant line 1. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

The *DIN3* expression across the treatments for Scan Line 11 matched the expected pattern, with strong induction in lines 2, 5 and 7: 12.1, 7.6 and 5.6 times *DIN3* expression respectively, when treated with three hours of darkness at 20°C compared with the transformant line 2 light sample. This induction was inhibited in seedlings of all lines, which experienced three hours of darkness at 5°C whose *DIN3* expression was 1.2, 1.1 and 1.01 times that of basal expression. All dark-induced *DIN3* expression was significantly different from that of the transformant 2 light sample (fig. 3.6.11).

In those same seedlings, *DIN3::LUC* expression mirrored that of *DIN3* and with similar levels of expression. Three hours of darkness at 20°C elicited strong *DIN3::LUC* expression in transformant lines 2, 5 and 7: 7.2, 10.1 and 3.3 times basal respectively. The dark-cold inhibition of *DIN3::LUC* expression was seen in all lines, *DIN3::LUC* expression following the three hours of darkness at 5°C was 1.1, 2.4 and 0.7 times basal expression respectively (fig. 3.6.11).

Scan Line 11 exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treatment was combined with 5°C. This pattern was replicated in the *DIN3::LUC* expression and with similar expression levels. The Scan Line 11 mutation changes CGTGAT of the native sequence to GAATTC; a GATA cis-acting motif (GATA) was detected within the 6 base-pairs of the EcoRI substitution. The mutation in the version of *DIN3::LUC* expressed by the Scan Line 11 seedlings did not disrupt or eliminate the characteristic dark induction seen in the native *DIN3* expression.



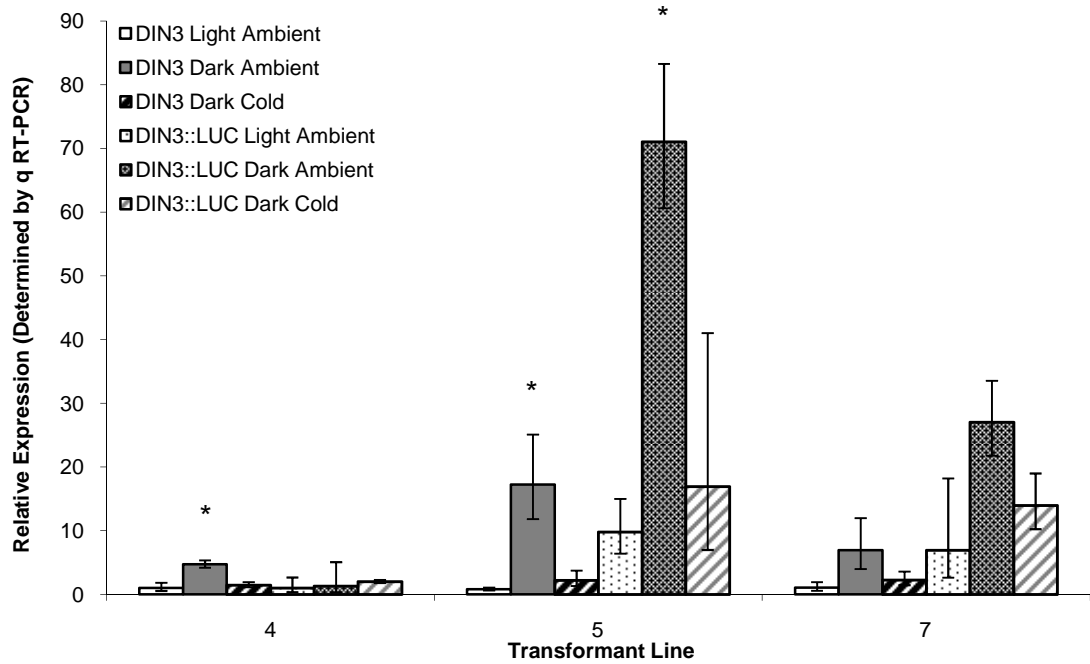
**Figure 3.6.11 *DIN3* and *DIN3::LUC* expression in 7-day-old Scan Line 11 seedlings following three hour light ambient, dark ambient and dark cold treatments**

Figure 3.6.11 Relative *DIN3* (solid colour) and *DIN3::LUC* (textured) expression levels in 7-day-old Scan Line 11 (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. Expression levels are relative to the light sample of transformant line 2. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

The *DIN3* expression across the treatments for Scan Line 12 matched the expected pattern, with strong induction in lines 4, 5 and 7: 4.7, 17.2 and 6.9 times *DIN3* expression respectively, when treated with three hours of darkness at 20°C compared with transformant line 4 light sample. This induction was inhibited in seedlings of all lines, which experienced three hours of darkness at 5°C whose *DIN3* expression was 1.5, 2.2 and 2.3 times that of basal expression. The dark-induced *DIN3* expression in transformant lines 4 and 5 was significantly different from that of the transformant 4 light sample (fig. 3.6.12).

In those same seedlings, *DIN3::LUC* expression broadly mirrored that of *DIN3*, three hours of darkness at 20°C elicited a strong induction of *DIN3::LUC* expression in transformant line 5 and 7: 71 and 27.1 times basal respectively. Three hours of darkness at 20°C elicited very weak induction of *DIN3::LUC* expression in line 4: 1.3 times basal and not statistically significant. With an unconfirmed dark-induction of *DIN3::LUC* expression in line 4, it was not possible to determine cold-inhibition in this line. The cold-inhibition of *DIN3::LUC* expression was seen in lines 5 and 7, *DIN3::LUC* expression following the three hours of darkness at 5°C was 16.9 and 13.9 times basal expression respectively. Only the dark-induced *DIN3::LUC* expression in transformant line 5 was significantly different from the basal expression (fig. 3.6.12).

Scan Line 12 exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treatment was combined with 5°C. This pattern was replicated in the *DIN3::LUC* expression of lines 5 and 7 but with the dark-induced *DIN3::LUC* expression at several times the *DIN3* expression level. The three hours of darkness at 20°C failed to induce *DIN3::LUC* expression in line 4 and neither could the presence of cold-inhibition of *DIN3::LUC* be determined. The Scan Line 12 mutation changes ATATTG of the native sequence to GAATTC; within the 6 base-pairs of the EcoRI substitution. With two out of the three transformant lines of Scan Line 12 tested exhibiting *DIN3::LUC* expression replicating the native *DIN3* expression pattern, the mutation in the version of *DIN3::LUC* expressed by the Scan Line 12 seedlings did not disrupt or eliminate the characteristic dark induction seen in the native *DIN3* expression.



**Figure 3.6.12 *DIN3* and *DIN3::LUC* Expression in 7-day-old Scan Line 12 seedlings following three hour light ambient, dark ambient and dark cold treatments**

Relative *DIN3* (solid colour) and *DIN3::LUC* (textured) expression levels in 7-day-old Scan Line 12 (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. Expression levels are relative to the light sample of transformant line 5. U.D. = Expression Undetermined i.e. so low as beyond the limits of detectability. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

When the entire results of the linker-scanning mutagenesis of the -300 to -250bp of the *DIN3* promoter is assembled (Table 3.6.1), it is evident that luminometry method, which confirmed whether *DIN3::LUC* was being expressed and whether a significant induction of *DIN::LUC* was seen after the 6h dark treatment, and the qRT-PCR, which directly monitored relative levels of *DIN3::LUC* expression follow 3h dark treatments at 20°C and 5°C, both provide results which accord with each other. The *DIN3::LUC* expression patterns of scan lines 2, 3, 4, 5, 6, 8 and 9 were not that seen in the *DIN3* promoter reporting lines or the native *DIN3* expression measured in the same sample of seedlings. The aberration in the *DIN3::LUC* expression patterns of these lines was the same – a loss of the characteristic induction of *DIN3::LUC* expression following a six or three dark treatment at 20°C. This fact prevented any meaningful analysis of the *DIN3::LUC* expression following the three dark treatments at 5°C via qRT-PCR. However it should be noted that never was there a loss of cold-inhibition of *DIN3::LUC* expression in those scan lines which exhibited a dark-induction. The linker-scanning mutation in those scan lines which exhibited aberrant *DIN3::LUC* expression did not always substitute part of or the entire sequence of a known cis-acting regulatory motif (Table 3.6.1). For example scan lines 2 and 9 exhibited aberrant *DIN3::LUC* expression but their mutations did not coincide with a known motif. Similarly, scan lines 7, 11 and 12 exhibited the characteristic expression patterns in response to the treatments, but expressed versions of *DIN3::LUC* with mutations which did coincide with known motifs.

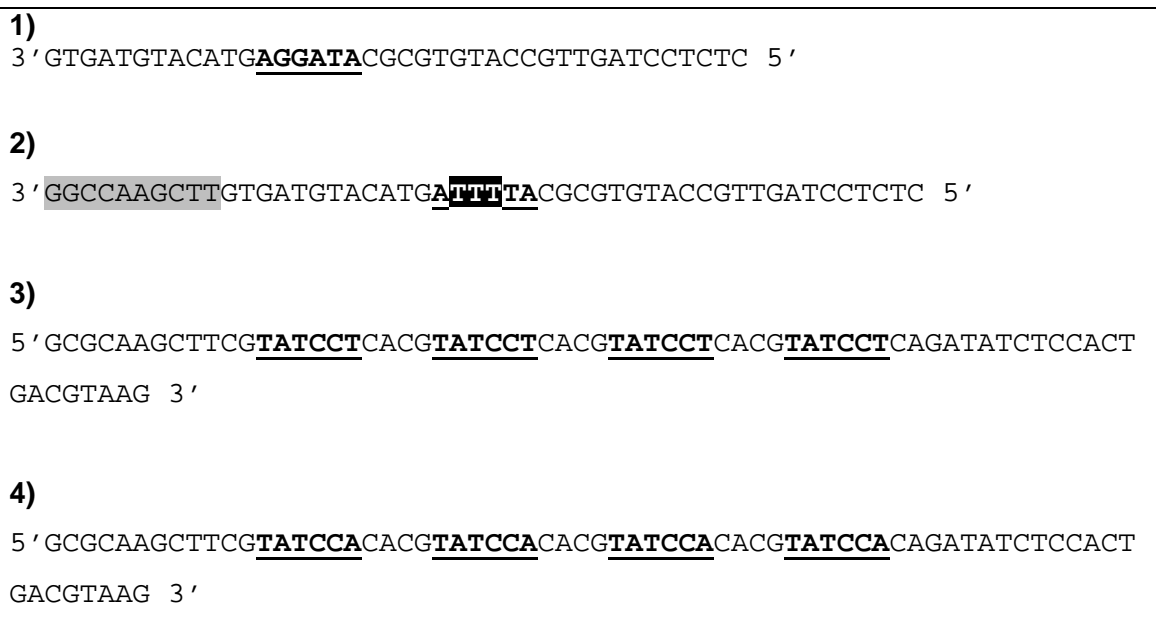
**Table 3.6.1 Summary of Linker-Scanning Mutagenesis results via luminometry and qRT-PCR**

A summary of the results of the linker-scanning mutagenesis of the essential -300 to -250bp of the *DIN3* promoter investigating possible cis-acting regulatory motifs which contribute towards the regulation of *DIN3* expression following dark and cold temperature treatments. 'Detectable *DIN3::LUC* expression' refers to the proportion of the transformant lines which exhibited levels of *DIN3::LUC* expression which was > 2 S.E.M. from the background. 'Significant Dark Induction' refers to the proportion of transformants expressing *DIN3::LUC* following a 6h dark treatment at levels > 2 S.E.M. from the light sample. 'Normal *DIN3* expression pattern' refers to the induction of *DIN3* expression following a 3h dark treatment at 20°C and its inhibition at 5°C as determined by qRT-PCR. 'Disrupted Motifs' refers to the number of known cis-acting regulatory motifs detected whose sequences were partly or entirely substituted by the EcoRI site present in the version of *DIN3::LUC* expressed by each scan line.

Scan Line	Luminometry		qRT-PCR			Disrupted Motifs
	Detectable <i>DIN3::LUC</i> Expression	Significant Dark Induction of <i>DIN3::LUC</i>	Normal <i>DIN3</i> expression pattern	Dark Induction of <i>DIN3::LUC</i>	Cold-inhibition of <i>DIN3::LUC</i>	
1	3/5 Transformants	2/5 Transformants	Yes	2/2 Transformants	2/2 Transformants	0
2	2/7 Transformants	1/7 Transformants	Yes	None	Unknown	0
3	2/8 Transformants	0/8 Transformants	Yes	None	Unknown	1
4	1/9 Transformants	0/9 Transformants	Yes	None	Unknown	3
5	0 Transformants	0 Transformants	No Data	No Data	No Data	3
6	1/9 Transformants	0/9 Transformants	Yes	None	Unknown	2
7	6/6 Transformants	4/6 Transformants	Yes	2/2 Transformants	2/2 Transformants	1
8	3/9 Transformants	0/9 Transformants	Yes	None	Unknown	1
9	5/7 Transformants	0/7 Transformants	Yes	None	Unknown	0
10	14/15 Transformants	10/15 Transformants	Yes	3/3 Transformants	3/3 Transformants	0
11	7/9 Transformants	4/9 Transformants	Yes	3/3 Transformants	3/3 Transformants	1
12	8/10 Transformants	7/10 Transformants	Yes	2/3 Transformants	2/3 Transformants	1

### 3.7 Analysis of putative dark/sugar responsive element

Three transgenic lines were created in order to investigate the role of a putative dark/sugar responsive element (TATCCT), previously referred to as MYBST1, in the -300bp portion of the *DIN3* promoter. Two different approaches were taken, the first involved the introduction of a -300bp *DIN3* promoter fused to a luciferase reporter where the TATCCT motif for the *DIN3* dark/sugar responsive element had been changed to TAAAAT, whilst leaving the rest of the promoter unmodified. The second approach was to create two concatamer lines (fig. 3.7.1), one using the hypothesised TATCCT *A. thaliana* sequence (Knight, H., unpublished data) for the *DIN3* dark/sugar responsive element (DARK1) and the second using the TATCCA *Oryza sativa* motif sequence for the *MYB3* dark/sugar responsive element (Lu et al., 1998) (DARK2). In both concatamers the elements were repeated four times attached to a 35S cauliflower mosaic virus (CaMV) minimal (90bp) promoter, which in turn was attached to the luciferase reporter gene (*luc<sup>INT</sup>*).



**Figure 3.7.1 Sequences of constructs for the putative dark/sugar responsive element**

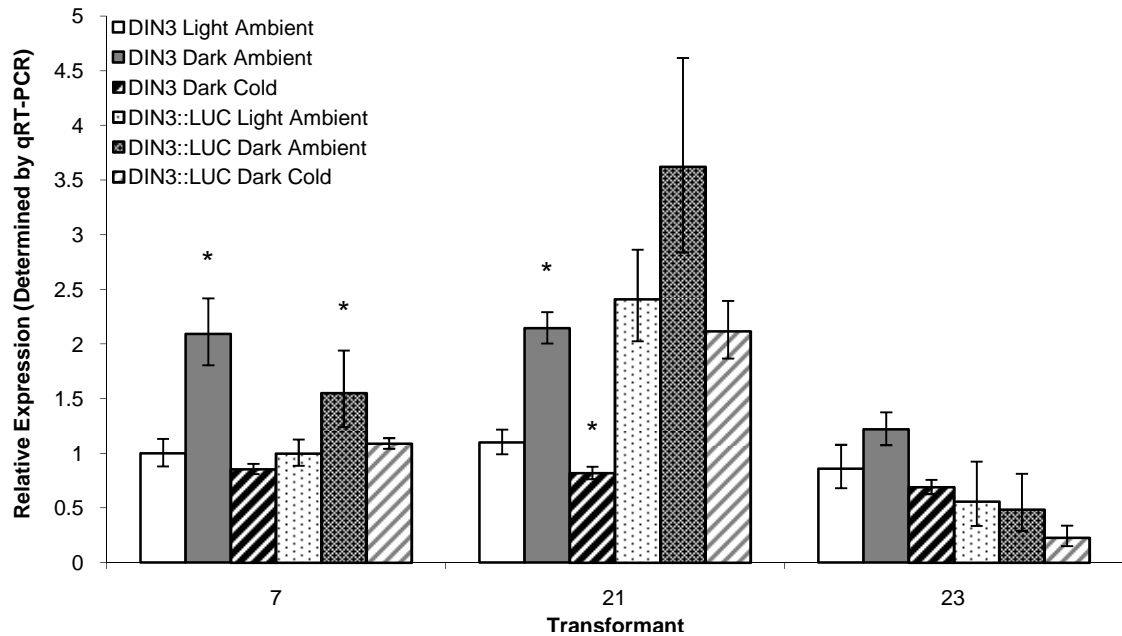
Relevant portions of the sequence of the constructs used for investigating the putative dark/responsive element: 1) the unmodified -300bp of *DIN3* promoter fused to *luc<sup>INT</sup>* (*DIN3::LUC*); 2) the version of *DIN3::LUC* with a mutated putative dark/responsive element; 3) concatamer of three TATCCT motifs fused to the 35S CaMV minimal promoter; 4) concatamer of three TATACCA motifs fused to the 35S CaMV minimal promoter. The relevant motifs are indicated by the bold and underlined nucleotides. In sequence 2 the grey highlighted sequence is the GC-clamp and restriction site; the mutation is indicated by the black highlighting and white text. Note that sequences 1 and 2 are shown in the anti-sense; therefore the sense site is changed from TATCCT to TAAAAT.

Seedlings of these lines were then grown to 7 days old and then underwent treatments of three hours of light or darkness at 20°C or darkness at 5°C. qRT-PCR was used to analyse their subsequent *DIN3* and *DIN3::LUC* or *luc<sup>INT</sup>* expression. Measuring *DIN3::LUC* expression permitted the investigation of what effect, the modified *DIN3* promoter had on the expression of the luciferase reporter gene (*luc<sup>INT</sup>*). Measuring the *luc<sup>INT</sup>* expression in the concatamer lines permitted the investigation of what role the two different putative dark/sugar responsive elements might have in the regulation of *DIN3*.

The *DIN3* expression across the treatments for *DIN3* TTT mutation line (figure 3.7.1) matched the expected pattern with clear dark-induction in transformant lines 7 and 21, both with 2.1 times *DIN3* expression; and weak dark-induction in line 23: 1.2 times *DIN3* expression, following three hours of darkness at 20°C, compared with transformant line 7 seedlings remaining in the light (basal). This induction was inhibited in seedlings of transformant lines 7, 21 and 23 which experienced three hours of darkness at 5°C whose *DIN3* expression was 0.9, 0.8 and 0.7 times that of basal expression respectively (fig. 3.7.2).

In those same seedlings, *DIN3::LUC* expression broadly mirrored that of *DIN3*. Three hours of darkness at 20°C induced *DIN3::LUC* expression in transformant lines 7 and 21: 1.6 and 3.6 times basal respectively; however in line 23 dark-induction was absent: 0.5 times basal. The dark-cold inhibition of *DIN3::LUC* expression was present in all lines; *DIN3::LUC* expression following the three hours of darkness at 5°C was 1.1, 2.1 and 0.2 times basal expression in lines 7, 21 and 23 respectively (fig. 3.7.2).

The *DIN3* TTT mutation line exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treated combined 5°C. This pattern was broadly mirrored by the *DIN3::LUC* expression. The three hours of darkness at 20°C induced *DIN3::LUC* expression in transformant lines 7 and 21 but failed to do so in line 23. The characteristic dark-cold inhibition of *DIN3::LUC* remained intact in all lines. The modified version of *DIN3::LUC* expressed by TTT was only disrupted in one out of three lines.



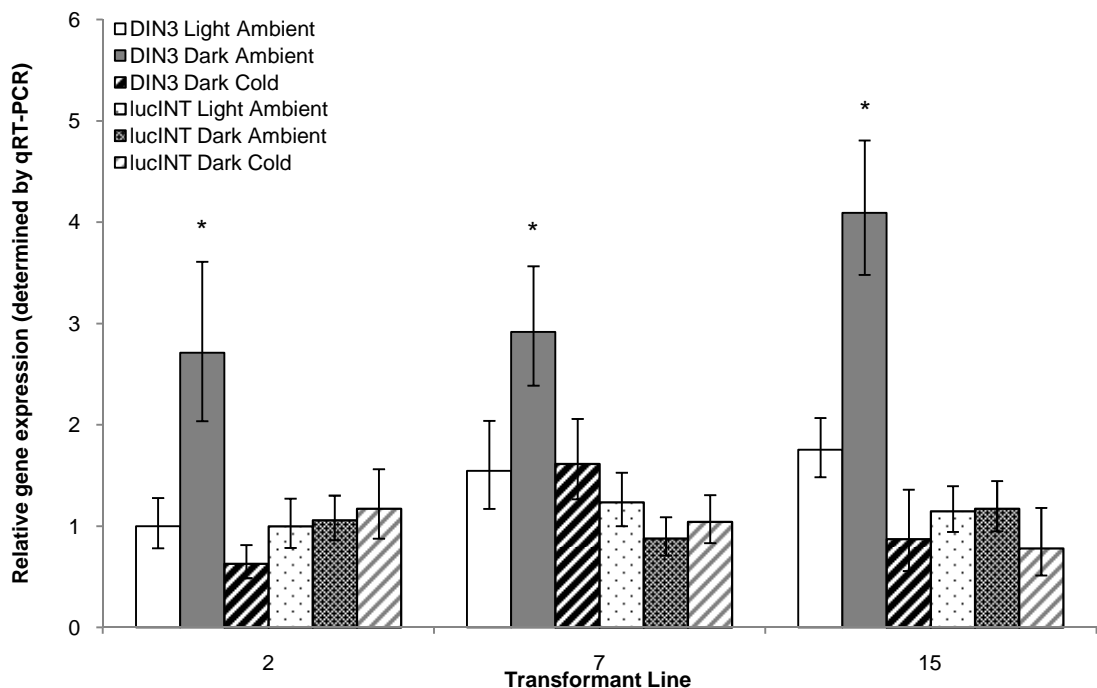
**Figure 3.7.2** *DIN3* and *DIN3::LUC* expression in 7-day-old TTT seedlings following three hour light ambient, dark ambient and dark cold treatments

Relative *DIN3* and *DIN3::LUC* expression levels in 7-day-old *DIN3* TTT mutation line (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. Expression levels are relative to the light sample of transformant line 7. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; *df*=4.

The *DIN3* expression across the treatments for Concatamer DARK1 matched the expected pattern with clear dark-induction in transformant lines 2, 7 and 15: 2.7, 2.9 and 4 times *DIN3* expression following three hours of darkness at 20°C, compared with transformant line 2 seedlings remaining in the light (basal). This induction was inhibited in seedlings of transformant lines 7, 21 and 23 which experienced three hours of darkness at 5°C whose *DIN3* expression was 0.6, 1.6 and 0.9 times that of basal expression respectively (fig. 3.7.3).

In those same seedlings, expression of the *luc<sup>INT</sup>* did not match that of *DIN3*. After three hours of darkness at 20°C the DARK1 concatamer seedlings of transformant line 7 did not exhibit an induction in *luc<sup>INT</sup>* expression: 0.9 times the expression of the light treated seedlings of transformant line 2. Three hours of darkness at 20°C triggered only a very weak increase in *luc<sup>INT</sup>* expression relative to basal expression in lines 2 and 15: 1.1 and 1.2 times respectively, however no line had *luc<sup>INT</sup>* expression significantly exceeding their light sample. Without clear dark-induction, the presence of dark-cold inhibition of expression could not be determined, but in none of the lines was dark-cold expression significantly greater than the light sample (fig. 3.7.3).

DARK1 concatamer seedlings exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treatment was carried out at 5°C. This pattern was absent in the *luc<sup>INT</sup>* expression. After three hours of darkness at 20°C the DARK1 concatamer seedlings did not exhibit *luc<sup>INT</sup>* expression significantly greater than expression following the light treatments; therefore the any dark-cold inhibition of expression could not be determined. The DARK1 concatamer containing three TATCCT motifs did not regulate the expression of the *luc<sup>INT</sup>* in the manner of *DIN3* following the three hour treatments.



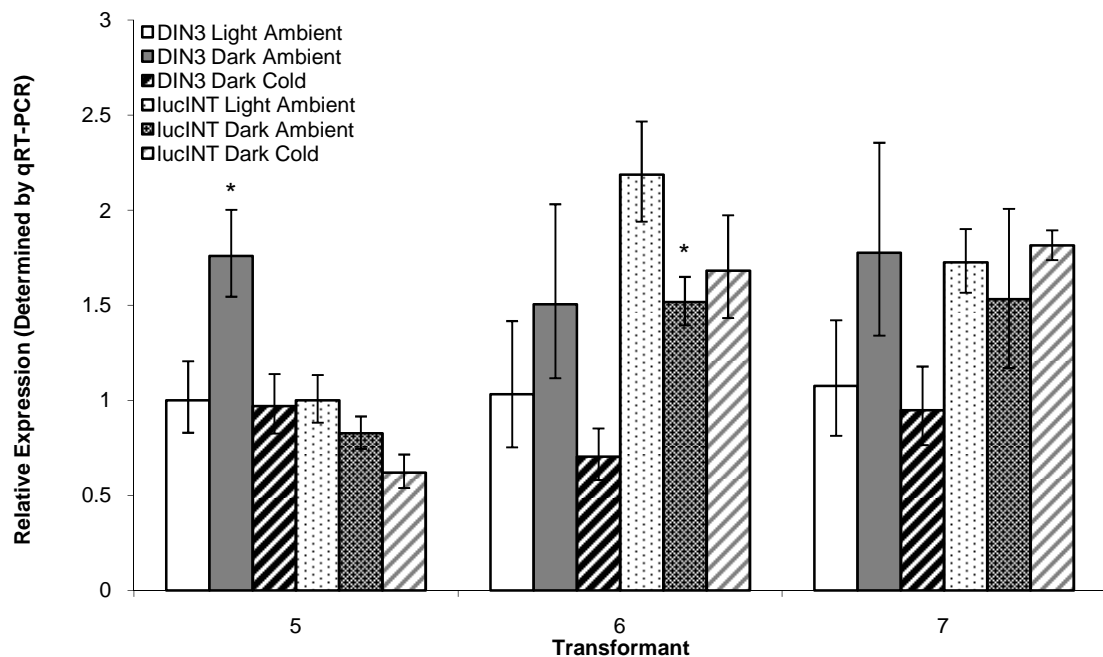
**Figure 3.7.3 *DIN3* and *luc<sup>INT</sup>* expression in 7-day-old DARK1 Concatamer seedlings following three hour light ambient, dark ambient and dark cold treatments**

Relative *DIN3* and *luc<sup>INT</sup>* expression levels in 7-day-old DARK1 (TATCCT) concatamer (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Expression levels are relative to the light sample of transformant line 2. Error bars represent the Student's *t*-distribution maxima and minima; df=7.

The *DIN3* expression across the treatments for concatamer DARK2 matched the expected pattern with dark-induction in transformant lines 5, 6 and 7: 1.8, 1.5 and 1.8 times *DIN3* expression following three hours of darkness at 20°C, compared with transformant line 5 seedlings remaining in the light (basal). This induction was inhibited in seedlings of transformant lines 5, 6 and 7 which experienced three hours of darkness at 5°C whose *DIN3* expression was 0.97, 0.7 and 0.95 times that of basal expression respectively (fig. 3.7.4).

In those same seedlings, *luc<sup>INT</sup>* expression did not match that of *DIN3*. After three hours of darkness at 20°C there was no induction of *luc<sup>INT</sup>* expression in transformant line 5: 0.8 times basal and only weak induction in lines 6 and 7: both 1.5 times basal. In all cases, their light-ambient expression was higher: 1, 2.2 and 1.7 times basal respectively. Without clear dark-induction of *luc<sup>INT</sup>* expression, the dark-cold inhibition, following the three hours of darkness at 5°C, could not be determined (fig. 3.7.4).

Concatamer DARK2 exhibited the characteristic *DIN3* expression pattern with an induction in *DIN3* expression following three hours at 20°C and an inhibition of this effect when the dark treated combined 5°C. This pattern was absent in the *luc<sup>INT</sup>* expression. After three hours of darkness at 20°C the DARK2 concatamer none of the seedlings exhibited *luc<sup>INT</sup>* expression significantly greater than expression compared light treatments, in fact all exhibited lower expression; therefore any dark-cold inhibition of expression could not be determined. The DARK2 concatamer containing three TATCCA motifs did not regulate the expression of the *luc<sup>INT</sup>* in the manner of *DIN3* following the three hour treatments.



**Figure 3.7.4 *DIN3* and *luc<sup>INT</sup>* expression in 7-day-old DARK2 Concatamer seedlings following three hour light ambient, dark ambient and dark cold treatments**

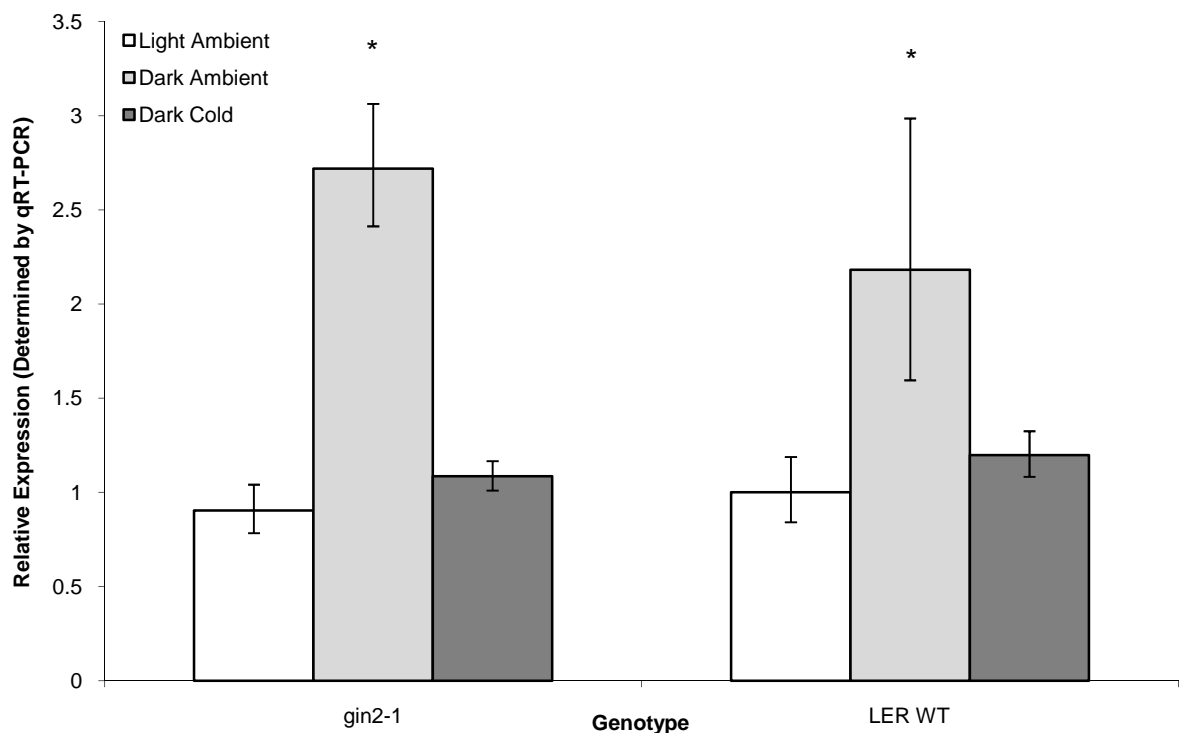
Relative *DIN3* and *luc<sup>INT</sup>* expression levels in 7-day-old DARK2 (TATCCA) concatamer (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (grey) or dark cold (5°C) (striped) treatments. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Expression levels are relative to the light sample of transformant line 5. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

Neither the mutation of the putative dark/sugar responsive element in the *DIN3::LUC* line nor the two concatamer lines, DARK1 and DARK2, indicated any significant role for this motif in the regulation of the *DIN3* response to the three hour dark treatment. Two out of three of the transformant lines of seedlings expressing *DIN3::LUC* with the mutation of the putative dark/sugar responsive element, exhibited an induction in the expression of *DIN3::LUC* after three hours of darkness at 20°C and an inhibition of this effect when the dark treatment was carried out at 5°C, thereby mirroring the native *DIN3* response to the treatments. All of the transformant lines of seedlings expressing the concatamer of TATCCT (DARK1) or TATCCA (DARK2) attached to the 35S CaMV minimal promoter and fused to *luc<sup>INT</sup>* did not exhibit patterns *luc<sup>INT</sup>* expression in the manner of the native *DIN3* expression. Based on these experiments, no evidence was found supporting a role of the putative dark/sugar responsive element acting alone in the regulating the expression of *DIN3* in response to prolonged darkness.

### 3.8 *DIN3* expression in plants insensitive to sugars via hexokinase

The current view of the literature gives the hexokinase-1 (HXK1) a central role in the regulatory mechanism of the *DIN* genes (Fujiki et al., 2000; Moore et al., 2003; Rolland et al., 2006). HXK1 acts as sensor of sugar state, specifically glucose, mediating the transduction of this signal to the nucleus. This elicits the up-regulation of transcription factor genes, which in turn regulate the expression of various sugar responsive genes, including the *DINs* (§1.1.3). Evidence for this mechanism comes from work carried out by Fujiki et al., (2000), whose experiments sought to indicate a mechanism by which the repression of *DIN* gene expression was mediated. Confirming the primacy of sugar over light in bringing about *DIN* gene repression, they proposed a role for the hexokinase pathway as the mechanism through which signals of sugar state are transduced and subsequently repress *DIN* gene expression. Experiments described earlier (§3.3) found that the mean glucose concentration of seedlings kept at 20°C was significantly greater than those at 5°C for three hours; there was no difference between the light and dark treatments (fig. 3.3.1). However the mean sucrose concentration was significantly higher in those seedlings keep in light at 5°C for three hours, compared with those in the dark at 5°C (fig. 3.3.2). To test this theory further, an experiment was designed using the *gin2-1* mutant line, which is a null-mutant for hexokinase-1 (HXK1) and so does not produce detectable levels of the enzyme (Moore et al., 2003). The *gin2-1* mutant line is in the Landsberg (Ler-0) ecotype and so seedlings of *gin2-1* were grown on 1% MS agar under long day conditions (16h:8h/light:dark) at 20°C for 7 days alongside Ler-0 wild-type. At 7 days old, the seedlings were treated to either 3h of light or darkness at 20°C or darkness at 5°C, before harvest and measurement of *DIN3* expression analysis by qRT-PCR. The rationale of the experiment was thus: the *gin2-1* seedlings lacking HXK1, which is not compensated by HXK2 (Moore et al., 2003), will be unable to sense a reduction in available sugar brought about by sudden and unexpected period of darkness within the entrained photoperiod. If *DIN3* relies on the HXK1 pathway for detecting sugar levels, then it would be expected that in the HXK1 null-mutant, *gin2-1*, the normal induction of *DIN3* after three hours of darkness would be absent due the lack of a sensory mechanism necessary to detect the change in sugar state.

The characteristic dark induction and cold repression of *DIN3* expression was observed in both the *gin2-1* mutant seedling and wild-type. The *DIN3* expression seen in the three hour dark treated plants kept at 20°C increased by 3 and 2.18 times in *gin2-1* and wild-type, respectively, compared to that of their corresponding light samples. In both cases this induction of *DIN3* expression was found to be significantly different to their light samples (fig. 3.8.1). Induction of *DIN3* expression in *gin2-1* following the 3h dark treatment at 5°C was inhibited, matching that of wild-type.



**Figure 3.8.1 *DIN3* expression in 7-day-old *gin2-1* and wild type *Arabidopsis* seedlings following three hour light ambient, dark ambient and dark cold treatments**

Relative *DIN3* expression in 7-day-old *Arabidopsis* (Ler-0) *gin2-1* and wild type (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (light grey) or dark cold (5°C) (dark grey) treatments. Expression levels are relative to the light sample of wild type. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

Although the *gin2-1* mutant seedlings lacked a functioning HXK1 pathway, the response in their *DIN3* expression to the dark treatments at 20°C and 5°C matched that of the wild-type seedlings. This was the typical response to those treatments previous characterised in the Columbia ecotype (fig. 3.6.1), that is: a significant induction in *DIN3* expression following three hours of darkness at 20°C compared to those remaining in the light and a inhibition or repression of this response when the dark treatment is carried out at 5°C. This experiment demonstrates that HXK1 is not required

for normal regulation of *DIN3* by light and low temperature. This is despite data indicating that glucose concentration is elevated in cold treated seedlings within three hours and that sucrose concentrations decline in seedlings which are kept in the dark for three hours at 5°C. The experiments conducted by Fujiki et al., (2000) all applied sugar treatments externally, leaf discs were suspended in solutions of different sugar concentrations. The implications of these findings, which seem to go against the conclusions made by the current literature, will be explored in the discussion.

## 4.1 Sequencing of transgenic and mutant lines

A number of different transgenic and mutant lines were used in this project. In a number of cases the sites of the mutations or T-DNA insertions (pAC161 vector) required confirmation by sequencing. Verification of constructs by sequencing was also required where it had not been done previously. A number of TILLING and T-DNA insertion lines of the *SRMYB* transcription factor gene were used to study the role of transcription factors. The TILLING lines were obtained from the *Seattle TILLING Project* (<http://tilling.fhcrc.org/>) and are lines generated by Ethyl Methanesulphonate (EMS) mutagenesis and screened for mutations in various genes. The loci of these mutations was listed in the TILLING Project database, however it required confirmation. Sequencing the gene in which these mutations occur will provide the exact loci of the mutations and their type. Likewise with the T-DNA insertions lines, (obtained from GABI-Kat, Universität Bielefeld) the exact locus of the T-DNA insertion found in line 516B12 had not been confirmed at the start of this project. Sequencing of the *SRMYB* gene within this line would identify the T-DNA locus. Binary vector overexpression plasmids carrying transcription factor genes were transfected into *Agrobacterium* thence used to transform *Arabidopsis* in order to overexpress these transcription factors in whole plants.

A PCR was carried using full length cDNA, synthesised from RNA of 7-day-old seedlings of *SRMYB* TILLING lines N94748, N90558 and wild type. The single base substitution mutations created by EMS in exons have the potential to alter the amino acid sequence, whereas if the mutation lies within an intron has the potential to alter splicing. If the intron mutation does affect splicing a different transcript length will be expected when the extracted RNA is transcribed to cDNA and a PCR carried out. It was hoped that the PCR would reveal any differences in splicing of the *SRMYB* gene in the TILLING lines compared to wild type, resulting from their intron mutations.

A 1% (w/v) agarose gel, stained with EtBr, was used to visualise the approximate the size of the products (fig. 4.1.1). The pair of gene-specific primers (primers 119 and 120; see Materials and Methods: Table 2.x for sequences) used for the *SRMYB* gene should produce a product with a theoretical maximum size of 1498bp, covering the majority of the coding region of the *SRMYB* gene. A pair of primers designed to genes *TUB1 – 4* (Knight et al., 1999) to act as an endogenous control produce a theoretical maximum

product size of 928bp. Two bands can be seen for each of the three templates, one larger, fainter band probably around 1100bp in size and the other around 980bp. The former is band is a portion of *SRMYB* gene and the latter that of the *TUB1 – 4* genes using primers TUB\_F and TUB\_R (Table 2.7.3.1). The tubulin band for all three templates is of the same intensity; this provides evidence that each reaction had the same starting amount of template (fig. 4.1.1) The bands for the *SRMYB* gene appear, as far as the gel resolution allows, to be almost identical in size (fig. 4.1.1). This suggests that there are no significant differences in the splicing of the transcripts of the *SRMYB* TILLING lines N94748, N90558 compared to wild type.(Dunn et al., 1998)



**Figure 4.1.1 Gel electrophoresis of PCR using cDNA to detect splicing differences.**

Image of a 1% agarose gel electrophoresis of PCR products using primers to the *SRMYB* (M) and *TUB1 – 4* (T) genes. The PCR templates were full length cDNA synthesised from RNA extracted from 7-day-old *SRMYB* TILLING lines N94748 and N90558 compared alongside that of Columbia 0 wild-type (Col 0). Bioline HyperLadder I was used as a size ladder (scale in base-pairs).

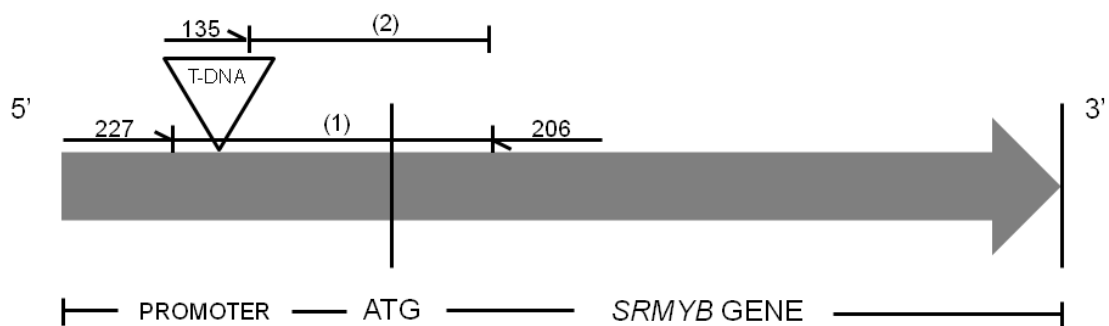
In previous work (Knight, H., unpublished), and again in this project via a semi-quantitative PCR analysing *SRMYB* gene expression in 7-day-old seedlings of *SRMYB* T-DNA insertion lines 516B12 and 783B02 and wild type (data not shown), it was found that seedlings of 783B02 had *SRMYB* gene that was completely knocked-out by the T-DNA insertion. However these results were not a quantitative assessment of gene expression, therefore a qRT-PCR analysis was conducted (fig. 4.5.1).

The location of the T-DNA insertions within the *SRMYB* gene was available from GABI-Kat (<http://www.gabi-kat.de/db/picture.php?genecode=At5g47390>) (Li et al., 2007); but required confirmation. Previous work in our laboratory confirmed the site of the insertion in 783B02 as in the third exon (Knight, H., unpublished). However the location of the T-DNA insertion in 516B12 was yet to be confirmed. The location of the T-DNA insertion is useful in better interpreting the results of analysis carried out into the expression levels of *SRMYB* in the 516B12 plants. Since *SRMYB* transcript of the expected length was detected in the 516B12 plants by PCR, it was hypothesised that the coding region was not interrupted by an insertion, hence the T-DNA insertion was likely to reside upstream of the coding region somewhere within the promoter. This was contrary to the annotation given on the GABI-Kat database, which predicted the insertion to be shortly downstream of the ATG start codon. A PCR was set up using combinations of primers, one (206) designed 220bp downstream of the ATG another (227) 659bp upstream into the promoter and another specific to the sequence of the T-DNA insertion (135) (fig. 4.1.2) in the aim of producing a product which could be sent for sequencing.

The PCR products were visualised on a 1% (w/v) agarose gel, stained with EtBr, to approximate the size and quantity of the products against *Bioline* HyperLadder I (fig. 4.1.3). The aforementioned primers were used in the following two combinations of pairs: 135 with 206 and 227 with 206, which give two products of distinctly different size. The pair 227 and 206 should give a theoretical maximum size of 882bp. Without knowing the location of the T-DNA insertion, it was impossible to predict the exact size of the product of a PCR using primers 135 and 206. Alongside the genomic DNA of 516B12, the two genomic DNAs of Col 0 wild type were used as controls. No band was produced for the 516B12 DNA using primers 227 and 206, which suggested that the T-DNA insertion laid somewhere, in between the primer binding sites of 227 and 206 (fig. 4.1.3: lane 1). Bands of the anticipated size were received for the wild type DNAs using

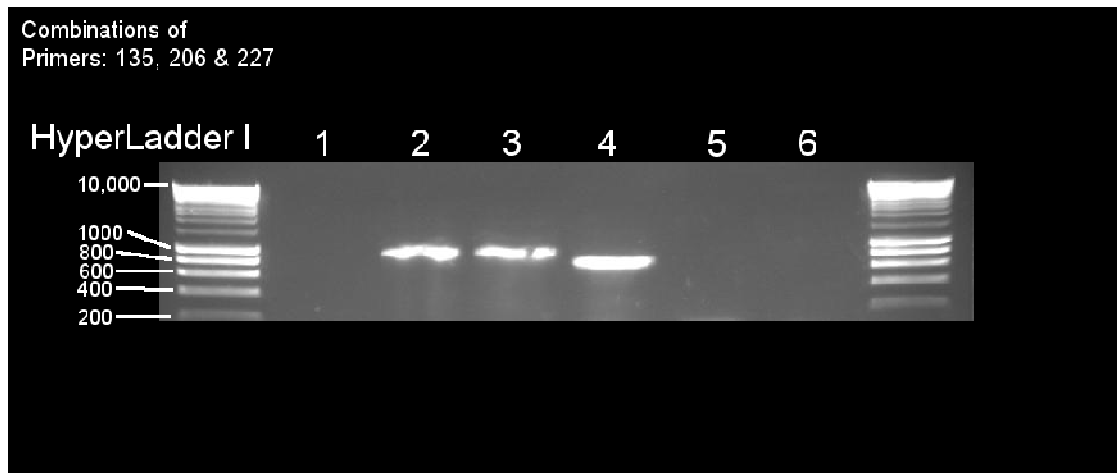
primers 227 and 206 (fig. 4.2.1.3: lanes 2 & 3). A band of around of 800bp was acquired for 516B12 using primers 135 and 206, this band was of a produce which included part of the T-DNA insertion and the remainder of the promoter, into which it was spliced, upstream of the ATG and the 206 primer binding site (fig. 4.1.3: lane 4). No bands were produced for the wild type DNAs using the 135 and 206 primers (fig. 4.1.3: lanes 5 & 6).

The 800bp band amplified from 516B12 was extracted using the *Qiagen* MinElute Gel Extraction Kit (*Qiagen Ltd.*, Crawley, UK) and the purified DNA sent for sequencing so that the exact location of the T-DNA could be determined.



**Figure 4.1.2 Schematic *SRMYB* gene map with annotations of relevant features**

A schematic of the hypothesised and confirmed location of the T-DNA insertion (inverted triangle) within the promoter of the *SRMYB* gene (grey arrow) of the 516B12 T-DNA insertion line. The relative locations of binding sites of primers 135, 206 and 227 are indicated by the single headed arrows labelled with the primer reference. The location of the ATG is included as a point of reference and separates the upstream promoter region from the downstream coding region. The portions of the *SRMYB* gene which would be amplified by a PCR using combinations of the primers are indicated by the numbered lines in between the primer binding sites. (1) is the product of primer pairs 227 and 206; (2) is the product of primer pairs 135 and 206.



**Figure 4.1.3 Gel electrophoresis of PCR using genomic DNA to map the location of the T-DNA insertion within the *SRMYB* gene of T-DNA line 516B12.**

Image of a 1% agarose gel electrophoresis of PCR products using primers to various positions of the *SRMYB* gene (fig. 4.2.1.3). Lane 1 using 516B12 DNA with primers 227 & 206; lanes 2 & 3 used Col 0 WT DNAs with primers 227 & 206; lane 4 used 516B12 DNA with primers 135 & 206; lanes 5 & 6 used Col 0 WT DNAs with primers 135 and 206. The PCR templates were genomic DNA extracted from 7-day-old *SRMYB* T-DNA lines 516B12 compared alongside that of two extractions of Columbia 0 wild-type (Col 0). Bioline HyperLadder I was used as a size ladder (scale in base-pairs).

Sequencing carried out by Heather Knight on the *SRMYB* gene possessed by the T-DNA line 783B02 revealed the location of the T-DNA insertion to be located within exon 3 (fig. 4.1.4). A primer designed downstream of exon 3 was used to sequence upstream into exon 3 revealing T-DNA sequence. The T-DNA insertion in the *SRMYB* gene of 783B02 does not occur within an intron, so is unlikely to deleteriously affect splicing of the gene following transcription. Such a large piece (~10kb) of foreign DNA will entirely disrupt the successful translation of the transcript or the assembly of the polypeptide as a functional protein. This hypothesis seems to be supported by the absence of a *SRMYB* transcript in 783B02 when analysed by PCR.

## AT5G47390.1 SRMYB

Genomic:

```
AAATAAAAAAAAAAATCCGGCCAGATAAAATCGAATTTATGTAATAAATCC
GACCAGATAAACTGATATTATTGTCTTTCTCCGCTCCTTTGTCTCTCTA
TCTCTTTCTCACAATTAGATTCTGTGCTTCTTCTGCGATCAACTAAGATC
CGATCCGCGAGCGTTTCAGACTTCGATCAGATCCGATTAAGAGAAGCAAA
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CACCGAAGGTTTCGATCCGGAAAAAGTGCAAGTATGGGTAATCTTAGCCATT
ACACGGGTTCTGGATCGGGTGGGCATGGAACCGGGTCCAACACTCCGGGT
TCTCCGGGTGATGTCCCTGACCATGTCGCTGGTGTGGTTACGCTTCTGA
GGATTTCTGTTGCTGGCTCTTCTCTAGCCGCGAGAGAAAAGAAAGTATCT
TCGTTTGATTTCTGAGATTAATTTTTTATCAAATCCAAATTTTTGTAA
TTGAGTTTATTTGCATCAAAGTCGTTGATTGCATTATGTAACAAGTGGT
GATCTGGTTTATGTAACAAGATTTTGTATGTGTGTTTGTATTTGGTTTTGT
TGTAGGAACCTCCATGGACAGAGGAAGAACACAGGATGTTCTTATTAGGTT
TACAGAAGCTGGGTAAAAGGTGATTGGAGAGGTATCTCAAGAAACTATGTG
ACCACTAGGACACCTACACAAGTTGCTAGCCATGCTCAGAAGTATTTTCAI
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TGGTTCCTGATGAGGTTTGTTCCTTCTTCATTCAAAAACACCATTTTTAT
TTATATTGGAGTGGTTACAAAATGTGTTGAGATACTGATTTAAAGGATTC
AGAAGCTTATTAGGTGGATTGGTTTGCCTTCTACATTTCAATATGAAAAG
TTGAAGTCTGTTGGGGTTCTAATTGATATGCTTGAGGATATCATTTTTGTA
GCCAATCCTGCTTAAGCATTTTTGGTCTTCTCATGGGAATGTGATCTTGAA
ATGTAATTTCTTTTCTTTATTCTGCTTATGCTGTGTGATTTGTCCTTGT
GGTTGGAGATATTCCCATGGATTTGCAAGAACCAGAGGAAGATAAATATTC
CTGTGAAAACGAAATGCAAGGTGCTGACTCTATTCATCAACACTTGCT
CCTAGCTCACTTACGCACCGTCAATCTTGAAATCGAAGAATGTGAATC
AATGGACTCCAAAACCTTACCACCGGGGAACCAACCGCAACTGCCGCTG
CTGCTTCTTCTTCTTCCAGACTAGAAGAAACCACACAACGCAATCACAA
CTGCAACCGCAGCCGCAACTACCTGGCTCATTCCCCATACTATATCCGAC
CTACTTTTACCATATTACCCGTTTCCATTCCCAATATGGCCTGCTGGTT
ATGTTCTGAACCACCAAGAAAGAGGAAACTCATGAAATTTCTCAGACCA
ACTGCTGTGCACTCGAAAGCTCCTATCAATGTTGACGAGCTTCTTGGTAT
GTCTAAGCTCAGCCTTGCAAGTCCAACAAAACATGGAGAATCCGATCAGT
CTCTTTTCAATGAAGCTAGGTGGCGGGTCATCTTCAAGACAATCAGCATT
CACCCGAATCCTAGCTCTGATAGTTTCAAGACATAAAAAGCGTGATACACGG
TTTATAAAGACCTGAGGAAGTGATGGTCTAAATGGGATCTGGTTTTGGG
GTTTACAGGTTAGTTGTTGGTCAAGTAACTTAAATAAGTTTTTTCTTTGT
TAGTTGTTTAACTTGGGTAGGATGTTTTAGTTTCAAGCTTTGATCATTAGG
GAAAAGAAAAAAGAAAAAAGGGAGAAAAACAAATTATTATTTTTTTG
CTTACATTTCTTTATATTTGTATGCTTTTATTTTACTCTAGGATGCGTT
AATTTTCGTTTAACTGTACTAAAAATTAGAATTTATTAGTTTGAATAA
ATAAAATCACAGTTTGT
```

EXON1

EXON2

EXON3

Part of EXON3 bound by TaqMan probe

BASE before which the T-DNA insertion is located

Primer binding site for sequencing into the EXON3 and the T-DNA insertion. TaqMan SRMYB probe binding site spans Exons 2 and 3. Underlined section of Exon 2 indicates the site. The grey highlighted sequence is the part of Exon 3 bound by the probe is duplicated following the Exon 2 sequence.

**Figure 4.1.4 Genomic sequence of AT5G47390.1: SRMYB of T-DNA line 783B02**

The annotated genomic sequence (obtained from TAIR) of (AT5G47390.1) the SRMYB of T-DNA line 783B02 indicating the location of the T-DNA insertion (pink base), the three exons (exon 1 – yellow; exon 2 – red; exon 3 – green), the primer binding site for sequencing (blue underlined text).

Following the sequencing of the 516B12 PCR product (fig. 4.1.3), the returned sequence was submitted to the TAIR BLAST to quickly determine how much the product aligned with the gene and crucially at which point it departed from this alignment. As was hypothesised from the results earlier PCR (fig. 4.1.3), the T-DNA insertion lies upstream of the ATG in the promoter region of *SRMYB* (fig. 4.1.5). Without knowing the location of important regulatory motifs within the promoter of *SRMYB* it is hard to say whether the position of the T-DNA is particularly deleterious to the regulation of the gene. However given the position of large piece of foreign DNA close to the start codon, any regulation provided by native promoter, now upstream of the T-DNA insertion, will be lost. The regulation of the gene will now be controlled by the new promoter in the form of the T-DNA and the remaining native promoter downstream of it, so undoubtedly the levels of gene expression will be altered significantly compared with wild-type. The effect of the T-DNA insertions on the *SRMYB* gene expression are considered later in the discussion.

## AT5G47390.1 SRMYB

Genomic:

```
AAATAAAAAAAAAAATCCGGCCAGATAAAATCGAATTTATGTAATAAATCC
GACCAGATAAACTGATATTATTGTCTTTCTTCCGCTCCTTTGTCTCTCTA
TCTCTTTCTCACAAATTAGATTCTGTGCTTCTTCTGCGATCAACTAAGATC
CGATCCGCGAGCGTTTCAGACTTCGATCAGATCCGATTAAGAGAAGCAAA
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CAGACAATCCAATGTCTCTCGTCGAAAAGACGTTCTAGTCTCTTTTGATA
TGGTTCCTGATGAGGTTTGTTCTTCTTCATTCAAAAACACCATTTTTAT
TTATATTGGAGTGGTTACAAAATGTGTTGAGATACTGATTTAAAGGATTC
AGAAGCTTATTAGGTGGATTGGTTTGCCTTCTACATTTCAATATGAAAAG
TTGAAGTCTGTTGGGGTTCTAATTGATATGCTTGAGGATATCATTTTTGTA
GCCAATCCTGCTTAAGCATTTTGGTCTTCTCATGGGAATGTGATCTTGAA
ATGTAATTTCTTTCTTTATTCTGCTTATGCTGTGTGATTTGTCCTTGTA
GGTTGGAGATATTCCCATGGATTTGCAAGAACCAGAGGAAGATAAATATTC
CTGTGGAAACTGAAATGCAAGGTGCTGACTCTATTCATCAGACACTTGCCT
CCTAGCTCACTTACGCACCGTCAATCTTGGAAATCGAAGAATGTGAATC
AATGGACTCCACAACTCTACCACCGGGGAACCAACCGCAACTGCCGCTG
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ACTGCTGTGCACTCGAAAGCTCCTATCAATGTTGACGAGCTTCTTGGTAT
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CTCTTTCATTGAAGCTAGGTGGCGGGTCATCTTCAAGACAATCAGCATTT
CACCCGAATCCTAGCTCTGATAGTTCAGACATCAAAAAGCGTGATACACGC
TTTATAAAAGACCTGAGGAAGTGATGGTCTAAAATGGGATCTGGTTTGGG
GTTTACAGGTTAGTTGTTGGTACAGTAACTTAAATAAGTTTTTCTTTGT
TAGGTTGTTTAACTTGGGTAGGATGTTTTAGTTCAGCTTTGATCATTAGG
GAAAAGAAAAAAGAAAAAAGGGAGAAAAACAAATTTATTTTTTTG
CTTACATTTCTTTATATTTGTATGCTTTTATTTTACTCTAGGATGCGTT
AATTTTCGTTTAACTGTACTAAAAATTAGAATTTATTTAGTTTTGAATAA
ATAAAATCACAGTTTGT
```

EXON1

EXON2

EXON3

Part of EXON3 bound by TaqMan probe

**BASE** before which the T-DNA insertion is located

Primer binding site for sequencing into the EXON3 and the T-DNA insertion. TaqMan *SRMYB* probe binding site spans Exons 2 and 3. Underlined section of Exon 2 indicates the site. The grey highlighted sequence is the part of Exon 3 bound by the probe is duplicated following the Exon 2 sequence.

### Figure 4.1.5 Genomic sequence of AT5G47390.1: *SRMYB* of T-DNA line 516B12

The annotated genomic sequence (obtained from TAIR) of (AT5G47390.1) the *SRMYB* of T-DNA line 783B02 indicating the location of the T-DNA insertion (pink base), the three exons (exon 1 – yellow; exon 2 – red; exon 3 – green), the primer binding site for sequencing (blue underlined text).

The only TILLING line mutation in the *SRMYB* detected by the sequencing of genomic DNAs (using gene-specific primers 119 & 120) of TILLING lines N90558, N92046 and N94640 was that present in N92046 (fig. 4.1.6). Despite the sequenced PCR product of N90558 covering the region between exons 1 and 3, no difference between the wild type *SRMYB* sequence and that of N90558 was found using TAIR BLAST. It may be that the TILLING line N90558 was incorrectly annotated by the *Seattle TILLING Project* database. From the sequencing results it appears that there is no intron mutation present in TILLING line N90558 as was described in the database. The described exon mutation present in TILLING line N92046 was localised to exon 3 (fig. 4.1.6) following the sequencing of its purified PCR product. TILLING line N92046 was described as possessing a silent exon mutation that is one which has no effect on the subsequent protein structure or function. TILLING line N94640 was described in the database as possessing a substitution mutation in an exon, following the sequencing of its purified PCR product no difference in its sequence compared to the wild type *SRMYB* sequence was identified using TAIR BLAST. The sequenced N94640 PCR product contained just a single base from exon 1 and around half of the sequence of exon 3. Clearly the sequence obtained was insufficient for the localisation of the N94640 mutation. The 119 and 120 primers start from the ATG of exon 1, in the case of primer 119, and end eight base-pairs upstream of the end of exon 3, in the case of primer 120. Another amplicon could have been produced from different combination of primers designed to amplify more of the 5' portion of the *SRMYB* gene. In addition an internal primer could have been used to sequence further upstream into exon 1 and two products sequenced and assembled to give a fuller assessment of the version of *SRMYB* found in N94640 and N90558.

```

>AT5G47390.1 | Symbols: | myb family transcription factor |
chr5:19226790-19228858 FORWARD
Length = 2069

Score = 1897 bits (957), Expect = 0.0
Identities = 969/972 (99%), Gaps = 1/972 (0%)
Strand = Plus / Plus

Query: 7 cttcgtttgatttctgagattaataatctttatcaaatccaaatcttgaattgagttt 66
      |||
Sbjct: 499 cttcgtttgatttctgagattaataatctttatcaaatccaaatcttgaattgagttt 558

Query: 67 attttgcatcaaagtcggtgattgcattatgtaacaagtggtgatctggttatgtaaca 126
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Sbjct: 559 attttgcatcaaagtcggtgattgcattatgtaacaagtggtgatctggttatgtaaca 618

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      |||
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Query: 187 cacaggatgttcttattaggtttacagaagctgggtaaaagtgattggagaggtatctca 246
      |||
Sbjct: 679 cacaggatgttcttattaggtttacagaagctgggtaaaagtgattggagaggtatctca 738

Query: 247 agaaactatgtgaccactaggacacctacacaagttgctagccatgctcagaagtatttc 306
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Sbjct: 739 agaaactatgtgaccactaggacacctacacaagttgctagccatgctcagaagtatttc 798

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Sbjct: 859 gatgaggtttgttctctctcattcaaaaacaccatttttattatattggagtggttac 918

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Sbjct: 1039 tatcattttgtagccaatcctgcttaagcattttggtctctctcatgggaatgtgacttg 1098

Query: 607 aaatgtaattctctcttattctgcttattgctgtgtgatttgccttgtaggttggag 666
      |||
Sbjct: 1099 aaatgtaattctctcttattctgcttattgctgtgtgatttgccttgtaggttggag 1158

Query: 667 atattcccatggatttgaagaaccagaggaagataatattcctgtggaactgaaatgc 726
      |||
Sbjct: 1159 atattcccatggatttgaagaaccagaggaagataatattcctgtggaactgaaatgc 1218

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Sbjct: 1219 aaggtgctgactctattcacaagactttgctcctagctcacttcacgcacogtcaatct 1278

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Sbjct: 1339 caactgcccgtgctgcttctctcttccagactagaagaaccacacaactgcaatcac 1398

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Query: 966 caccatattacc 977
      |||
Sbjct: 1459 caccatattacc 1470

```

**Figure 4.1.6 TAIR BLAST output for N92046 *SRMYB* against wild type AT5G47390.1: *SRMYB***

PCR product (QUERY) of N92046 genomic DNA using 119 and 120 *SRMYB* primers purified and sequenced then analysed using TAIR BLAST. Red ellipse indicates the mutation in the TILLING line present in exon 3.

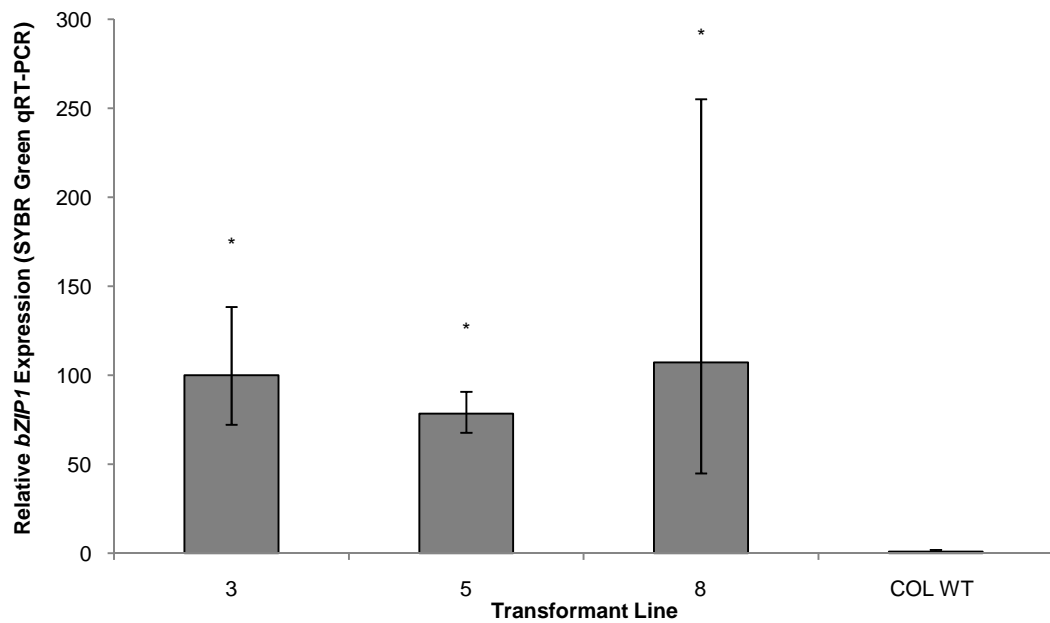
Prior to the transformation of *Agrobacterium* or *Arabidopsis* with a transgene, it is wise to check that the plasmid containing the transgene matches that in the database or the original design. All but one of the transcription factor overexpression plasmids were generated prior to the start of this project in our laboratory and had been sequenced (Ülker, B., unpublished) and verified as being correct. However the plasmid (obtained from Philip Gilmartin) containing the *GATA21* transcription factor gene had not. In this case, plasmid DNA was sent for sequencing and then checked against the sequence for *GATA21* found in the TAIR database using TAIR BLAST (fig. 4.1.7). Six misalignments were identified. Such errors will naturally cast doubt on any results obtained from using plants over-expressing the version of *GATA21* carried by this plasmid.



## **4.2 Confirmation of over-expression**

The seedlings which passed through the BASTA selection required further screening in order to confirm that they were truly overexpressing the transcription factor coded in the transgene. To achieve this, seedlings of these putative overexpressing lines were grown under long-day conditions to seven days old. Following RNA extraction and a full length cDNA synthesis, a PCR was carried out and visualised on an agarose gel to provide a rough semi-quantitative analysis of the expression levels of the transcription factor genes. In many cases the results were in consistent, but the consensus was used to pick transformant lines for further analysis by RT PCR. The results of these analyses are presently described.

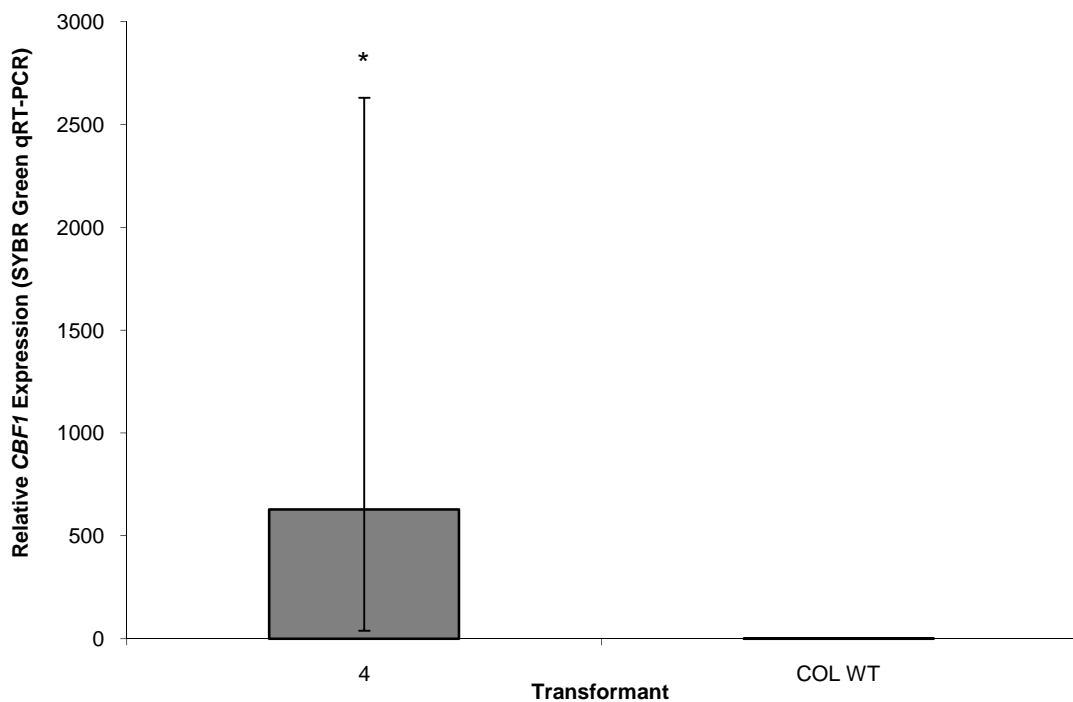
Initially, ten transformants (selected by BASTA spraying) were assayed for their *bZIP1* (AT5G49450) expression via PCR of cDNA and visualised on a gel. The results from which were not conclusive, but did indicate candidate over-expressing transformant lines; these were latterly analysed by qRT-PCR (SYBR Green) for definitive confirmation of *bZIP1* over-expression. Seedlings of transformant lines 3, 5 and 8 were 100.1, 78.6 and 107.2 times greater in their expression of *bZIP1* than Col 0 WT seedlings (fig. 4.2.1). Additionally the *bZIP1* expression of all the transformant lines was significantly different from the Col 0 WT expression.



**Figure 4.2.1 *bZIP1* expression in 7-day-old seedlings of putative overexpressors relative to Col 0 WT expression levels**

Relative *bZIP1* expression levels in 7-day-old Col 0 WT and putative *bZIP1* over-expressing (standardised against *PEX4* expression) seedlings grown under long-day (16h light / 8h dark) conditions at 20°C. Expression levels are relative to that of the Col 0 WT sample. The asterisk (\*) indicates those data sets which were outside the distribution of the Col 0 WT data set. Error bars represent the Student's t-distribution maxima and minima; df=4.

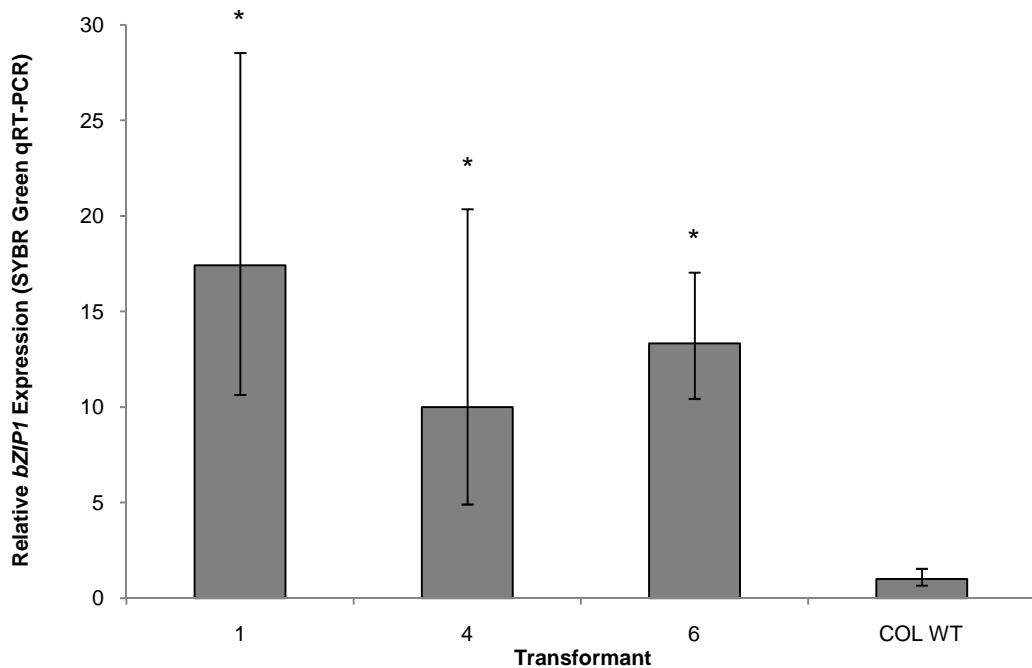
Initially, ten transformants (selected by BASTA spraying) were assayed for their *CBF1* (AT4G25490) expression via PCR of cDNA and visualised on a gel. The results from which were conclusive, indicating just a single over-expressing transformant line; line 4. This was latterly analysed by qRT-PCR (Taqman<sup>®</sup>) for definitive confirmation of *CBF1* over-expression. Seedlings of transformant line 4 were 628.5 times greater in their expression of *CBF1* than Col 0 WT seedlings (fig. 4.2.2). Additionally the *CBF1* expression of all the transformant lines was significantly different from the Col 0 WT expression.



**Figure 4.2.2 *CBF1* expression in 7-day-old seedlings of putative overexpressors relative to Col 0 WT expression levels**

Relative *CBF1* expression levels in 7-day-old Col 0 WT and putative *CBF1* over-expressing (standardised against *PEX4* expression) seedlings grown under long-day (16h light / 8h dark) conditions at 20°C. Expression levels are relative to that of the Col 0 WT sample. The asterisk (\*) indicates those data sets which were outside the distribution of the Col 0 WT data set. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

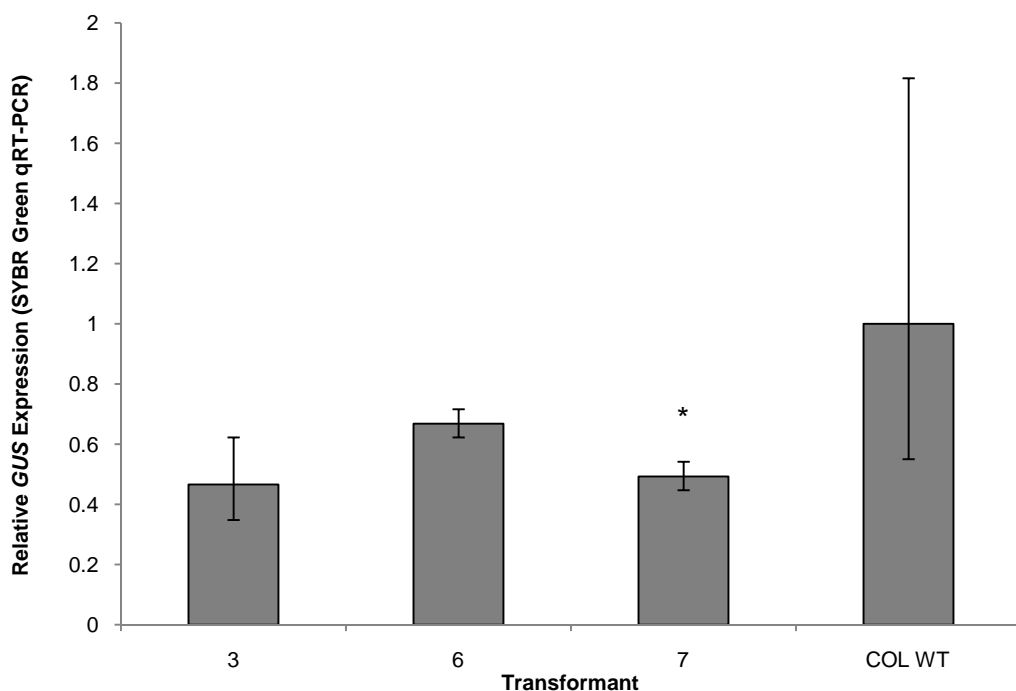
Initially, ten transformants (selected by BASTA spraying) were assayed for their *GATA21* (AT5G56860) expression via PCR of cDNA and visualised on a gel. The results from which were not conclusive, but did indicate candidate over-expressing transformant lines; these were latterly analysed by qRT-PCR (SYBR Green) for definitive confirmation of *GATA21* over-expression. Seedlings of transformant lines 1, 4 and 6 were 17.4, 10 and 13.3 times greater in their expression of *GATA21* than Col 0 WT seedlings (fig. 4.2.3). Additionally the *GATA21* expression of all the transformant lines was significantly different from the Col 0 WT expression.



**Figure 4.2.3 *GATA21* expression in 7-day-old seedlings of putative overexpressors relative to Col 0 WT expression levels**

Relative *GATA21* expression levels in 7-day-old Col 0 WT and putative *GATA21* over-expressing (standardised against *PEX4* expression) seedlings grown under long-day (16h light / 8h dark) conditions at 20°C. Expression levels are relative to that of the Col 0 WT sample. The asterisk (\*) indicates those data sets which were outside the distribution of the Col 0 WT data set. Error bars represent the Student's *t*-distribution maxima and minima; *df*=4.

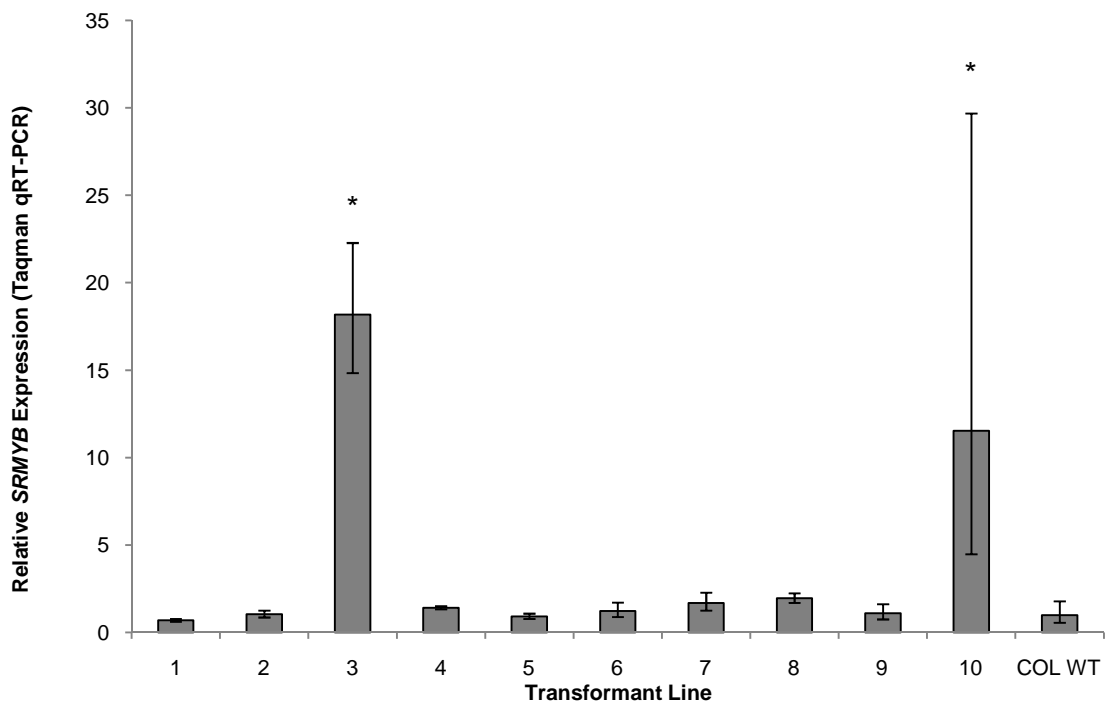
Plants were dipped with *A. tumefaciens* carrying a *35S::GUS* construct, transformants from which were to be used as controls against which to compare the effects of transcription factor overexpression. Initially, eight transformants (selected by BASTA spraying) were assayed for their *GUS* expression via PCR of cDNA and visualised on a gel. The results from which were not conclusive, but did identify candidate transformant lines; these were latterly analysed by qRT-PCR (SYBR Green) for definitive confirmation of expression of the *35S::GUS* gene. Seedlings of transformant lines 3, 6 and 7 expressed *GUS* at 0.47, 0.67 and 0.49 times that of Col 0 WT seedlings (fig. 4.2.4). In transformant line 7 this difference was significant. *GUS* is not found in wild-type so there should no *GUS* expression detected in the wild-type sample, and so its presence suggests that the level of *GUS* expression in the putative transformants is lower than what can be detected by qRT-PCR. Therefore analysis of *GUS* expressed in the putative *35S::GUS* transformants, revealed that none of the three lines selected from semi-quantitative PCR were true transformants, but false positives.



**Figure 4.2.4 *GUS* expression in 7-day-old seedlings of putative *35S::GUS* transformants relative to Col 0 WT expression levels**

Relative *GUS* expression levels in 7-day-old Col 0 WT and putative *35S::GUS* transformants (standardised against *PEX4* expression) seedlings grown under long-day (16h light / 8h dark) conditions at 20°C. Expression levels are relative to that of the Col 0 WT sample. Error bars represent the Student's *t*-distribution maxima and minima; *df*=4.

Ten transformants (selected by BASTA spraying) were assayed for their *SRMYB* (AT5G47390) expression by qRT-PCR (Taqman<sup>®</sup>) relative to Col 0 WT expression. Only transformant lines 3 and 10 had expression levels more than double that of Col 0 WT: 18.2 and 11.5 times respectively. Only with transformant line 10 was this difference in expression significantly from Col 0 WT (fig. 4.2.5). These analyses permitted the identification of which transformant lines were genuinely overexpressing the particular transcription factor.



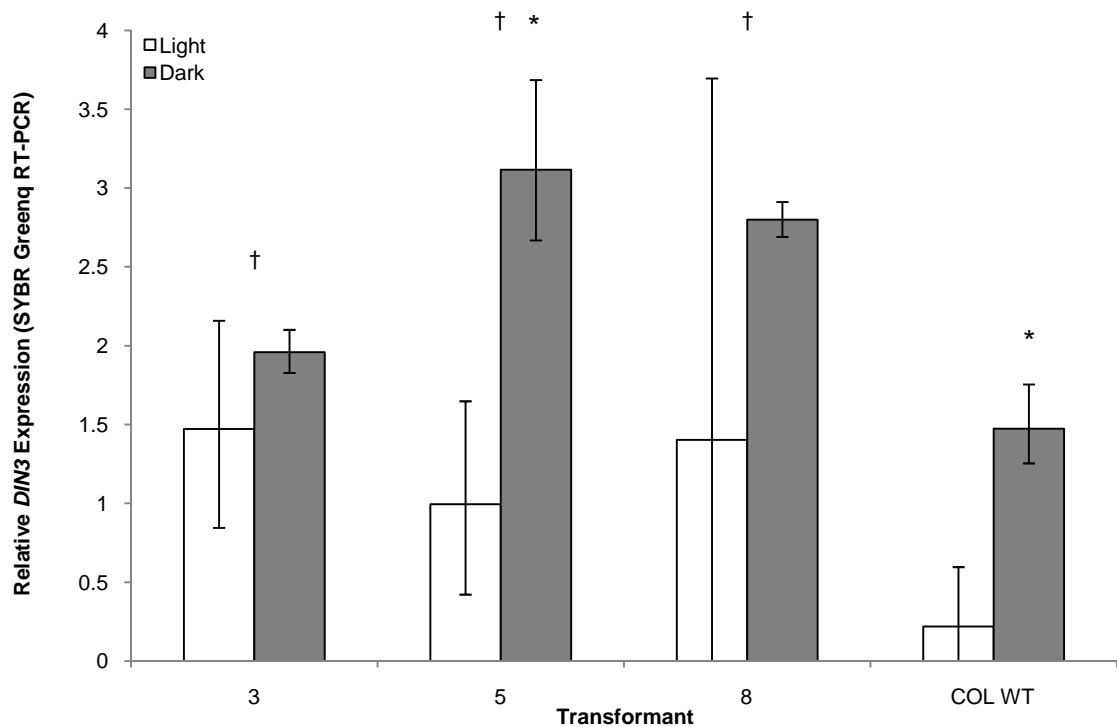
**Figure 4.2.5 *SRMYB* expression in 7-day-old seedlings of putative overexpressors relative to Col 0 WT expression levels**

Relative *SRMYB* expression levels in 7-day-old Col 0 WT and putative *SRMYB* over-expressing (standardised against *PEX4* expression) seedlings grown under long-day (16h light / 8h dark) conditions at 20°C. Expression levels are relative to that of the Col 0 WT sample. The asterisk (\*) indicates those data sets which were outside the distribution of the Col 0 WT data set. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

### **4.3 Effect of transcription factor over-expression on *DIN3* expression**

The transcription factors under investigation were chosen for their putative role in the regulating *DIN3* expression in response to changes in environmental conditions, namely extended darkness and chilling (§4.1). In order to test the hypothesis that these transcription factors indeed have a role in regulating *DIN3* expression, the transcription factors were overexpressed singly *in planta* (§2.4.4; §4.2 ) and then the transformants treated to three hours of light or darkness at 20°C or darkness at 5°C. The *DIN3* expression within the seedlings was analysed following the treatments to determine what, if any, effect the overexpression of the transcription factors had compared to wild-type. The results of these analyses are considered presently.

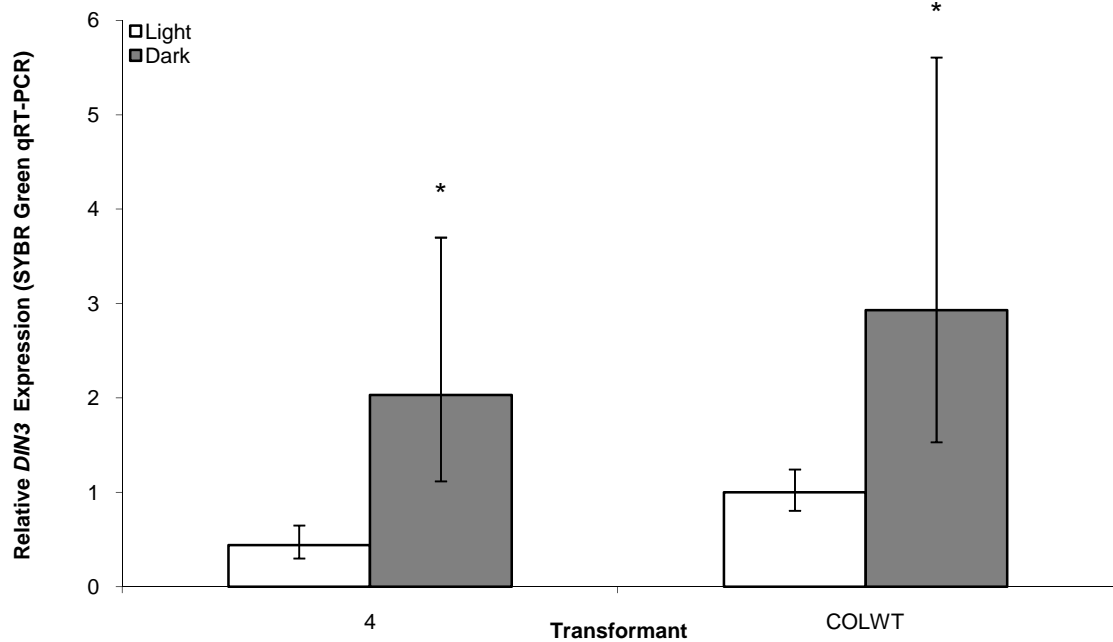
The *DIN3* expression in the three transformant lines over-expressing *bZIP1* (AT5G49450) demonstrated the characteristic dark induction, following three hours of darkness at 20°C. The *DIN3* expression of over-expressing lines 3, 5 and 8, following the three hour dark treatment, was 2, 3.1 and 2.8 times that of *DIN3* expression of the light sample of Col 0 WT. The *DIN3* expression of lines 3, 5 and 8 was 1.4, 2.1 and 1.9 times greater than the dark sample of Col 0 WT. In all cases these differences were significant (fig. 4.3.1).



**Figure 4.3.1 *DIN3* Expression in 7-day-old Col 0 WT and *bZIP1* over-expressing seedlings following three hour light and dark ambient treatments**

Relative *DIN3* expression levels in 7-day-old Col 0 WT and putative *bZIP1* over-expressing (standardised against *PEX4* expression) seedlings following 3h in light (white) or darkness (grey) at 20°C. Expression levels are relative to that of the Col 0 WT light sample. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light data set. The dagger (†) indicates those data sets which were outside the distribution of the Col 0 WT dark data set. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

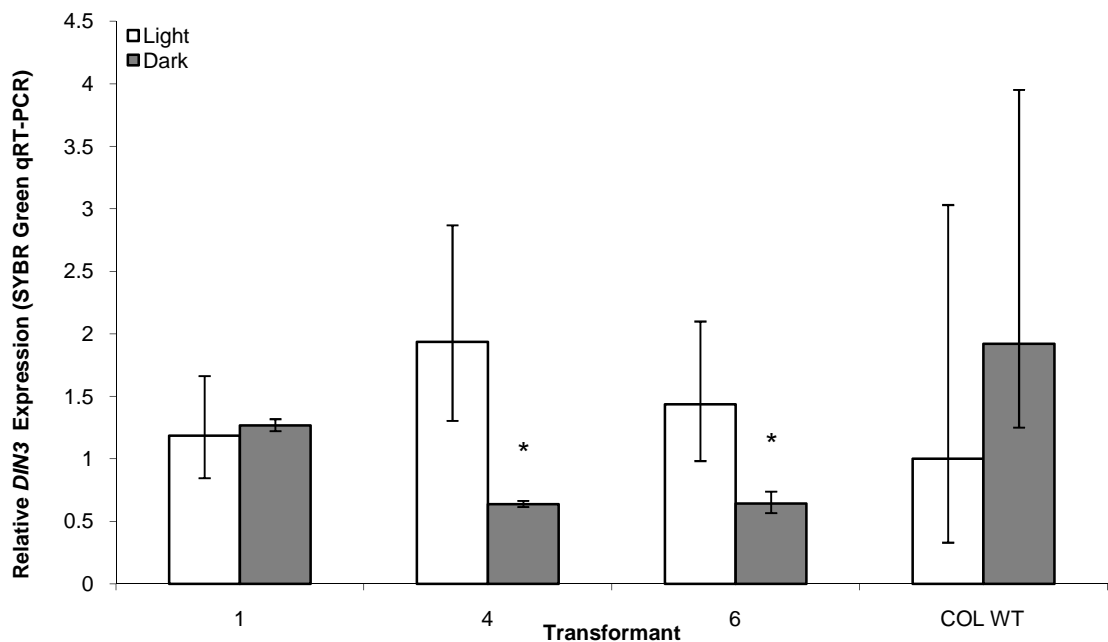
The *DIN3* expression in the transformant line over-expressing *CBF1* (AT4G25490) demonstrated the characteristic dark induction, following three hours of darkness at 20°C. The *DIN3* expression of over-expressing line 4, following the three hour dark treatment, was 2 times that of *DIN3* expression of the light sample of Col 0 WT. The dark *DIN3* expression of Col 0 WT was 1.4 times greater than that of *CBF1* over-expressing line 4. However this difference was not significant (fig. 4.3.2).



**Figure 4.3.2 *DIN3* Expression in 7-day-old Col 0 WT and *CBF1* over-expressing seedlings following three hour light and dark ambient treatments**

Relative *DIN3* expression levels in 7-day-old Col 0 WT and putative *CBF1* over-expressing (standardised against *PEX4* expression) seedlings following 3h in light (white) or darkness (grey) at 20°C. Expression levels are relative to that of the Col 0 WT light sample. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light data set. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

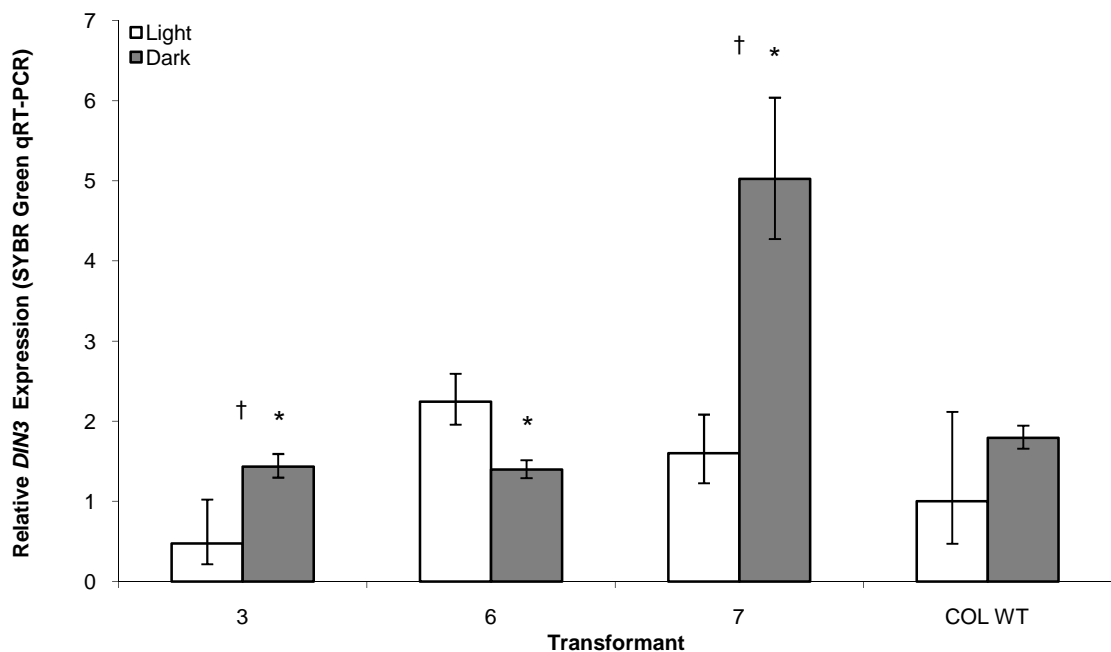
The *DIN3* expression in the three transformant lines over-expressing *GATA21* (AT5G56860) lacked the characteristic dark induction, following three hours of darkness at 20°C. The *DIN3* expression of over-expressing lines 3, 5 and 8, following the three hour dark treatment, was 1.3, and 0.6 times that of *DIN3* expression of the light sample of Col 0 WT respectively. However the *DIN3* expression of these lines following three hours in the light was 1.2, 1.9 and 1.4 respectively. The dark *DIN3* expression of Col 0 WT was 1.5 and 3 times greater than lines 3, 5 and 8 respectively. However none of these differences were significant (fig. 4.3.3).



**Figure 4.3.3 *DIN3* Expression in 7-day-old Col 0 WT and *GATA21* over-expressing seedlings following three hour light and dark ambient treatments**

Relative *DIN3* expression levels in 7-day-old Col 0 WT and putative *GATA21* over-expressing (standardised against *PEX4* expression) seedlings following 3h in light (white) or darkness (grey) at 20°C. Expression levels are relative to that of the Col 0 WT light sample. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light data set. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

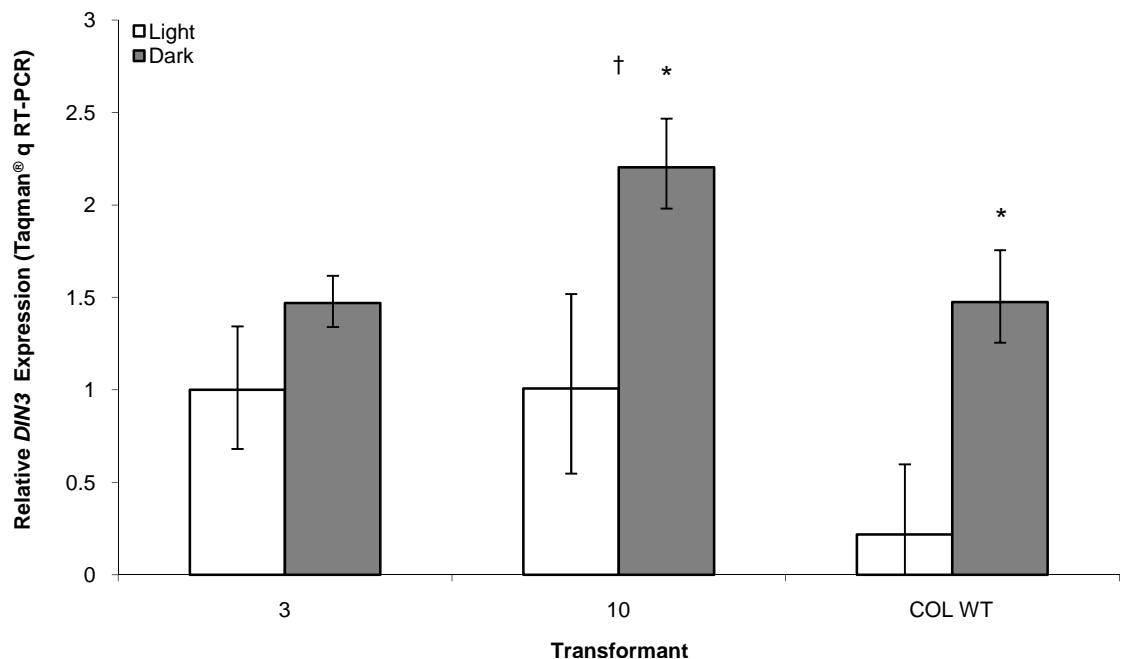
The *DIN3* expression in two out of the three putative *35S::GUS* transformant lines demonstrated the characteristic dark induction, following three hours of darkness at 20°C. The *DIN3* expression of putative transformant lines 3, 6 and 7, following the three hour dark treatment, was 1.4, 1.4 and 5 times that of *DIN3* expression of the light sample of Col 0 WT (constitutive). Notably line 6 lacked dark induction, with the light *DIN3* expression exceeding that of dark: 2.2 times wild-type expression. The dark *DIN3* expression of Col 0 WT was 1.2 and 1.3 times greater than lines 3 and 6 respectively. *DIN3* expression of line 7 was 2.8 times greater than the dark sample of Col 0 WT. However none of these differences were significant (fig. 4.3.4).



**Figure 4.3.4 *DIN3* Expression in 7-day-old Col 0 WT and *35S::GUS* expressing seedlings following three hour Light and Dark Ambient Treatments.**

Figure 4.3.4 Relative *DIN3* expression levels in 7-day-old Col 0 WT and putative *35S::GUS* expressing (standardised against *PEX4* expression) seedlings following 3h in light (white) or darkness (grey) at 20°C. Expression levels are relative to that of the Col 0 WT light sample. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light data set. The dagger (†) indicates those data sets which were outside the distribution of the Col 0 WT dark data set. Error bars represent the Student's *t*-distribution maxima and minima; *df*=4.

The *DIN3* expression in the three transformant lines over-expressing *SRMYB* (AT5G47390) demonstrated the characteristic dark induction, following three hours of darkness at 20°C. The *DIN3* expression of over-expressing lines 3, and 10, following the three hour dark treatment, was 1.4 and 2.2 times that of *DIN3* expression of the light sample of Col 0 WT. The *DIN3* expression of line 10 was 1.5 times greater than the dark sample of Col 0 WT. In line 10 the increase in *DIN3* expression, following three hours of darkness at 20°C, compared with Col 0 WT was significantly different (fig. 4.3.5).



**Figure 4.3.5 *DIN3* Expression in 7-day-old Col 0 WT and *SRMYB* over-expressing seedlings following three hour light and dark ambient treatments**

Figure 4.3.5 Relative *DIN3* expression levels in 7-day-old Col 0 WT and putative *SRMYB* over-expressing (standardised against *PEX4* expression) seedlings following 3h in light (white) or darkness (grey) at 20°C. Expression levels are relative to that of the Col 0 WT light sample. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light data set. The dagger (†) indicates those data sets which were outside the distribution of the Col 0 WT dark data set. Error bars represent the Student's *t*-distribution maxima and minima; *df*=4.

The analyses into the effects of the overexpression of certain transcription factors produced results permitting the identification of which these affected the expression of *DIN3*. Three independently transformed lines overexpressing *bZIP1* had significantly elevated *DIN3* expression compared to wild type. The induction of *DIN3* in response to three hours of darkness at 20°C was also present in these three lines. Seedlings overexpressing *CBF1* had slightly reduced expression of *DIN3* overall compared to wild type however this difference was not significant. Three independently transformed lines

overexpressing *GATA21* lost the characteristic induction of *DIN3* expression following a three hour dark treatment at 20°C as seen in the wild type seedlings. Instead they had elevated *DIN3* expression in the light compared to wild type and their own dark treated samples. Only in two lines was the difference between the light and dark treatments significant. Three independently transformed lines putatively expressing *35S::GUS* had no consistent *DIN3* expression pattern between them. Two out of three exhibited a dark induction and out of those two, one was expressed *DIN3* at significantly lower levels, the other at significantly higher levels. It is worth remembering that all these supposed *35S::GUS* expressing lines did so at lower levels than the wild type control (fig. 4.2.4). This result adds further doubt as to whether these seedlings were *35S::GUS* transformants at all. Two independently transformed lines overexpressing *SRMYB* exhibited the characteristic dark induction of *DIN3* expression following a three hour dark treatment at 20°C and did at a marginally higher magnitude than wild type. However this difference was only significant in one line.

## 4.4 Effect of the over-expression of transcription factors

Transcription factors play an essential role in gene regulation. Their effects can be subtle, which is best detected via gene expression analysis, or it may have a more pronounced and discernable effect on the phenotype. And so it is in this section that the results of monitoring the effects on the phenotypes, of transformant plants overexpressing transcription factor genes, will be described. The overexpressors (*bZIP1*: AT5G49450; *CBF1*: AT4G25490; *GATA21*: AT5G56860; *SRMYB*: AT5G47390), chosen for their potentially significant effects on light/dark responses, were previously analysed for changes in *DIN3* expression (§4.3). The growth regimes were designed to provide conditions under which the potential effects of the overexpression of the transcription factors might be seen. The overexpressors were grown initially on 1% MS agar plates under long day conditions (16h:8h/light:dark) at 20°C for 7 days, after which individual seedlings were transplanted to peat discs. Each transformant line was, at the point of transplantation, represented by 30 seedlings. 30 seedlings of Columbia 0 wild-type were also grown alongside for comparison. These 30 seedlings were divided into three batches of ten for two experiments.

### Effect on dark-induced chlorosis

Two batches of ten 7-day-old seedlings of each overexpressing line were grown for a further week under long-day conditions at 20°C, so that they were 14 days old at the time of the commencement of the experiment. One of these batches of 14-day-old seedlings was transferred to a greenhouse, with supplemented light to produce long day conditions and with a median daytime temperature of 23°C. The other batch was kept in a growth cabinet at 20°C, but deprived of light for 11 days.

### Effect on growth under short-day conditions

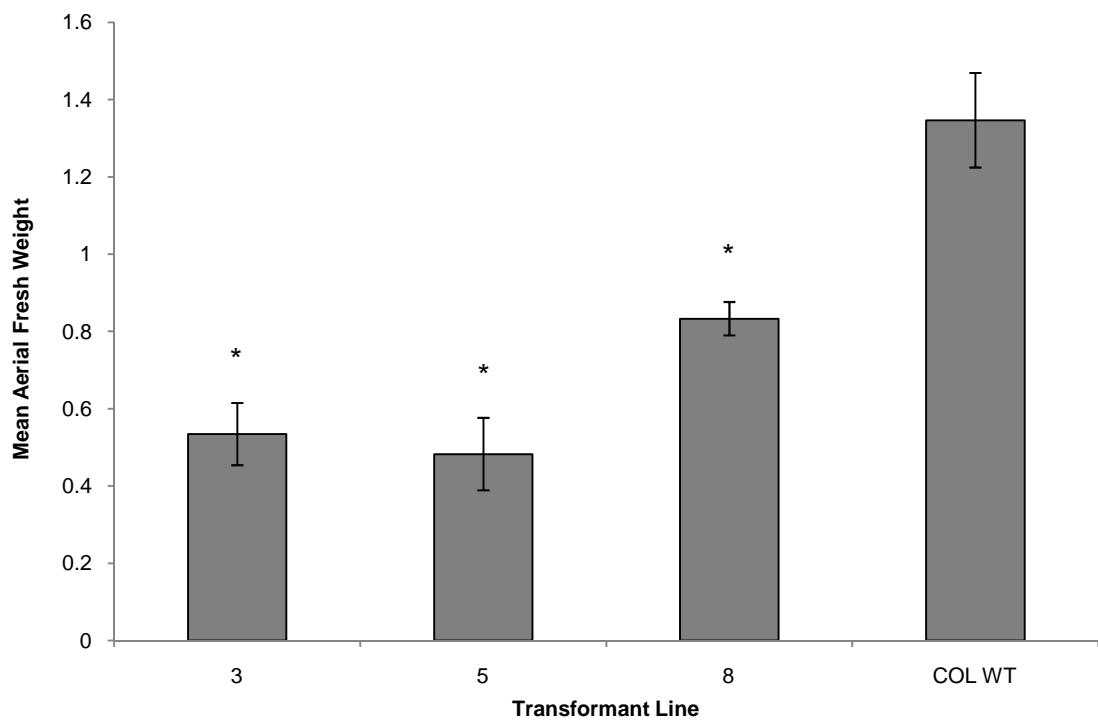
A batch of ten 7-day-old seedlings of each overexpressing line, was grown for seven weeks under short day conditions (8h:16h/light:dark) at 20°C. The short day grown plants were analysed quantitatively by measuring their aerial fresh weight and number of rosette leaves at the end of the 7 weeks. The aerial dry weight would have been a better indicator as to the efficiency of biomass production, however only the fresh weight was recorded. The number of leaves compared with the fresh weight provides a measure of development. Additionally, photographs were taken of each plant prior to harvest. This experiment provides a gross assessment of the effects of overexpressing

the various transcription factor genes and crucially determines whether transcription factor overexpression improves growth.

### Results of growth under short-days

After seven weeks grown under short day conditions, the mean aerial fresh weight of all the transformant lines of *A. thaliana* overexpressing *bZIP1* was lighter than the mean of the wild-type plants. The mean aerial fresh weight of transformant lines 3, 5 and 8 were 0.4, 0.36 and 0.62 times that of wild-type respectively, in all cases these differences were significant at the 99.9% confidence level as determined by a two-tailed Student's *t*-test (fig. 4.4.1).

Plants overexpressing *bZIP1* exhibit reduced biomass, producing around half the aerial biomass of wild-type plants when grown under short day conditions.

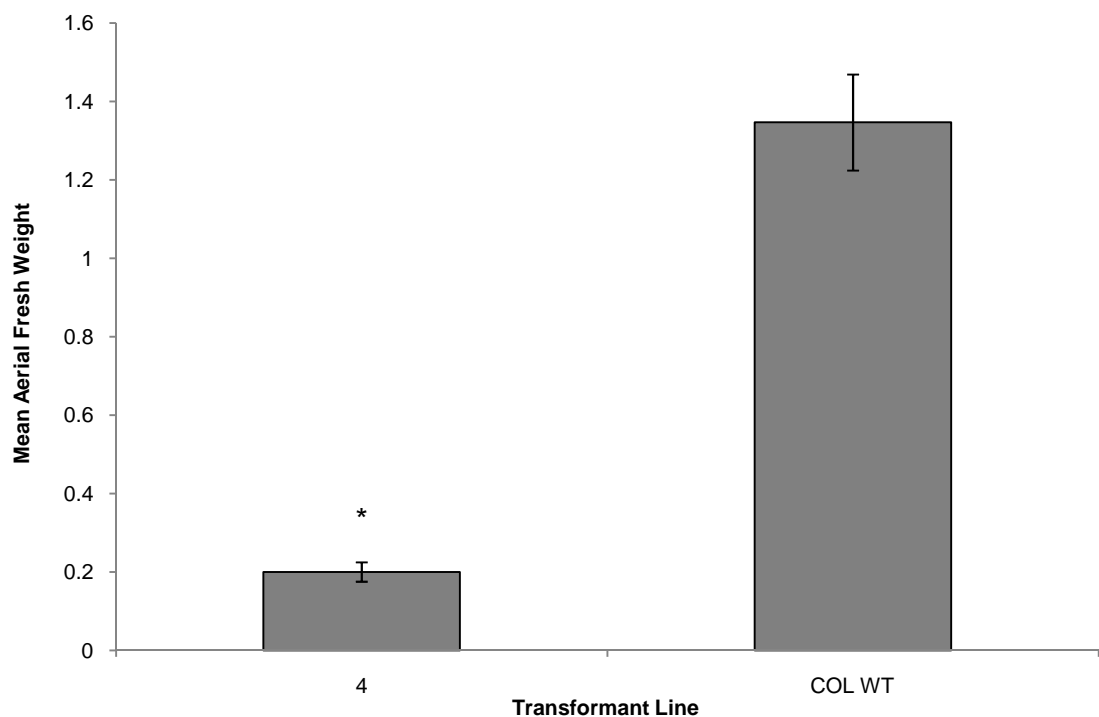


**Figure 4.4.1 Mean aerial fresh weight of 7-week-old *A. thaliana* plants overexpressing *bZIP1* compared to wild-type**

Grey bars represent the mean aerial fresh weight of short-day grown 7-week-old *A. thaliana* plants overexpressing *bZIP1* and Columbia 0 wild-type. Error bars represent the standard error of the mean. The asterisk (\*) indicates those transformant lines whose means were significantly different from Col 0 WT as determined by a two-tailed *t*-test (*t*-test results for transformant lines 3, 5 & 8 were all:  $p < 0.001$ ;  $df = 19$ ;  $t_{critical} = 2.1$ ).

After seven weeks grown under short day conditions, the mean aerial fresh weight of *A. thaliana* plants overexpressing *CBF1* was lighter than the mean of the wild-type plants. The mean aerial fresh weight of transformant line 4 – the only transformant – was 0.15 times that of wild-type, this difference was significant at the 99.9% confidence level as determined by a two-tailed Student's *t*-test (fig. 4.4.2).

Plants overexpressing *CBF1* produce less than one fifth the aerial biomass of wild-type plants when grown under short day conditions. However there was only one transformant line from which the plants overexpressing *CBF1* were derived.

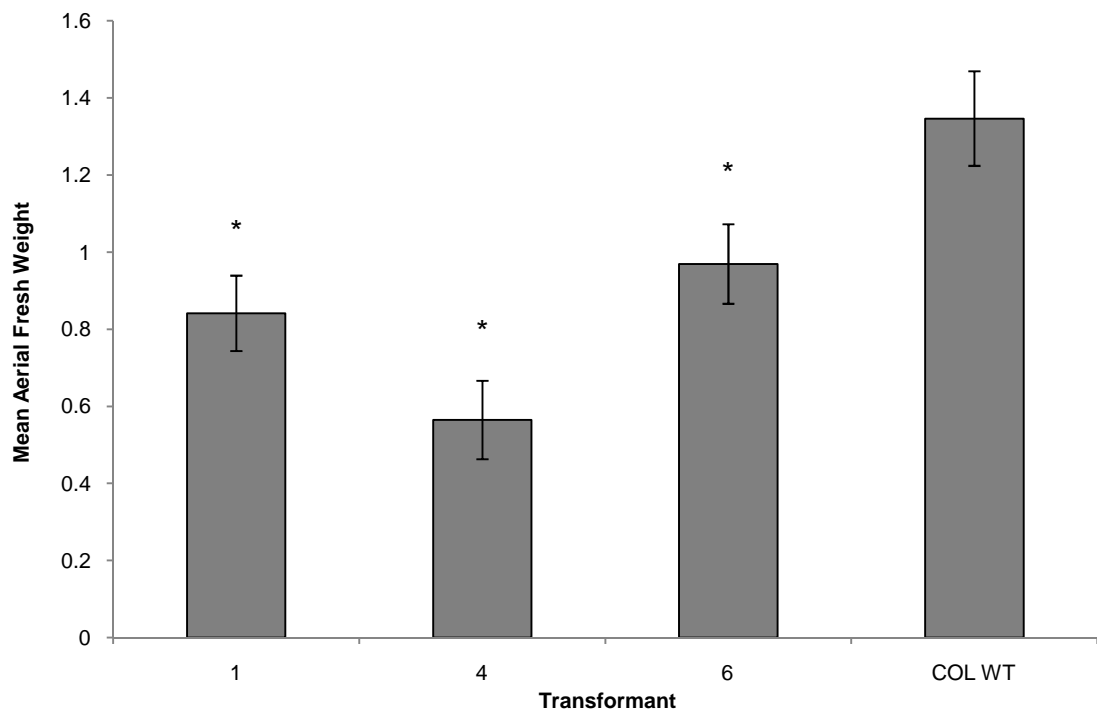


**Figure 4.4.2 Mean aerial fresh weight of 7-week-old *A. thaliana* plants overexpressing *CBF1* compared to wild-type**

Grey bars represent the mean aerial fresh weight of short-day grown 7-week-old *A. thaliana* plants overexpressing *CBF1* and Columbia 0 wild-type. Error bars represent the standard error of the mean. The asterisk (\*) indicates those transformant lines whose means were significantly different from Col 0 WT as determined by a two-tailed *t*-test (*t*-test result for transformant line 4 was:  $p < 0.001$ ;  $df = 18$ ;  $t \text{ critical} = 2.2$ )

After seven weeks grown under short day conditions, the mean aerial fresh weight of all the transformant lines of *A. thaliana* overexpressing *GATA21* was less than that of wild-type. The mean aerial fresh weight of transformant lines 1, 4 and 6 were 0.62, 0.42 and 0.72 times that of wild-type respectively, in all cases these differences were significant at the 99.9% confidence level as determined by a two-tailed Student's *t*-test (fig. 4.4.3).

Plants overexpressing *GATA21* exhibited a reduced biomass, producing less than two-thirds the aerial biomass of wild-type plants when grown under short day conditions.

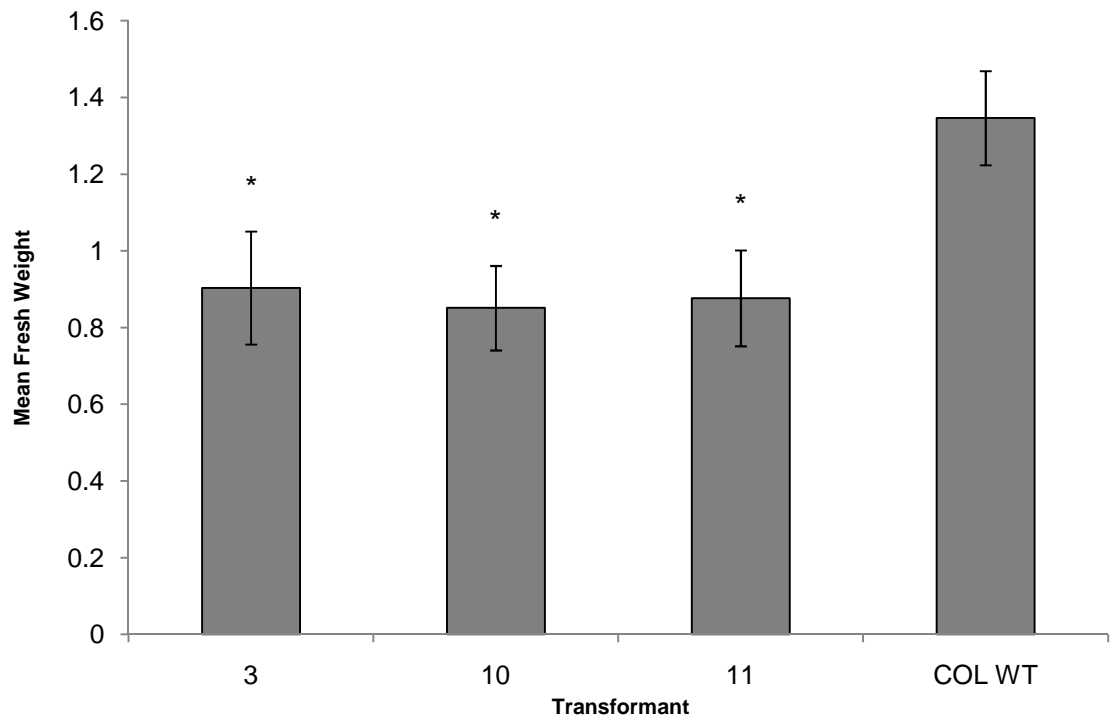


**Figure 4.4.3 Mean aerial fresh weight of 7-week-old *A. thaliana* plants overexpressing *GATA21* compared to wild-type**

Grey bars represent the mean aerial fresh weight of short-day grown 7-week-old *A. thaliana* plants overexpressing *GATA21* and Columbia 0 wild-type. Error bars represent the standard error of the mean. The asterisk (\*) indicates those transformant lines whose means were significantly different from Col 0 WT as determined by a two-tailed *t*-test (*t*-test results for transformant lines 1, 4 & 6 were all:  $p < 0.001$ ;  $df = 19$  (Transformant 6  $df = 18$ );  $t$  critical = 2.1).

After seven weeks grown under short day conditions, the mean aerial fresh weight of all the transformant lines of *A. thaliana* overexpressing *SRMYB* was less than that of wild-type. The mean aerial fresh weight of transformant lines 3, 10 and 11 were 0.67, 0.63 and 0.65 times that of wild-type respectively, in all cases these differences were significant at the 99.9% confidence level as determined by a two-tailed Student's *t*-test (fig. 4.4.4).

Plants overexpressing *SRMYB* exhibit reduced biomass, producing around two-thirds the aerial biomass of wild-type plants when grown under short day conditions. It is worth considering comparing the growth effects seen in the MYB mutants (§4.3.4) to these overexpressors. Two of the mutants had a mean aerial fresh weight significantly different from wild-type. The first, 516B12, has a T-DNA insertion the *SRMYB* (AT5G47390) promoter. This mutation did not create a significant change in the *SRMYB* expression levels in 516B12 plants compared to wild-type. The second N90558 has an unconfirmed intron mutation and had expression levels similar to wild-type. 783B02, a mutant with significantly reduced *SRMYB* expression levels, did not exhibit significantly different mean aerial fresh weight compared to wild-type. The overexpression results combined with those of the *SRMYB* mutant lines suggest that levels of *SRMYB* are important in plant productivity or growth rate, although it is unlikely to be correlated directly to absolute transcript abundance.

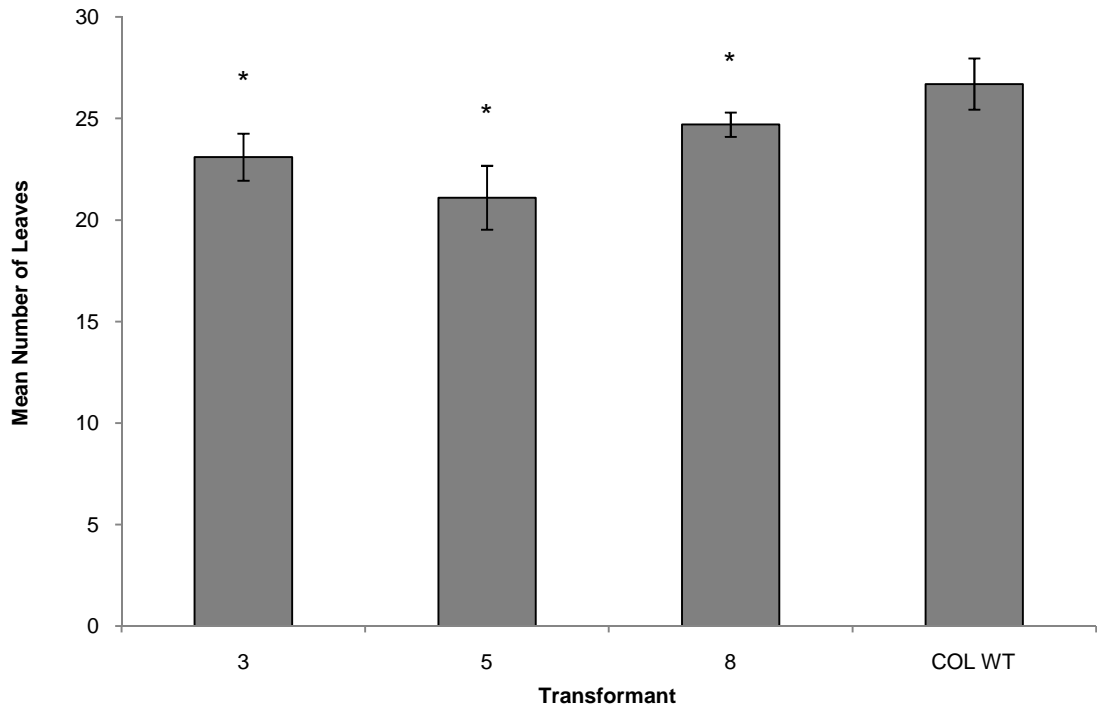


**Figure 4.4.4 Mean aerial fresh weight of 7-week-old *A. thaliana* plants overexpressing *SRMYB* compared to wild-type**

Grey bars represent the mean aerial fresh weight of short-day grown 7-week-old *A. thaliana* plants overexpressing *SRMYB* and Columbia 0 wild-type. Error bars represent the standard error of the mean. The asterisk (\*) indicates those transformant lines whose means were significantly different from Col 0 WT as determined by a two-tailed *t*-test (*t*-test results for transformant lines 3, 10 & 11 were all:  $p < 0.001$ ;  $df = 19$  (Transformant 3  $df = 17$ );  $t$  critical = 2.1).

After seven weeks grown under short day conditions, the mean number of rosette leaves of all the transformant lines of *A. thaliana* overexpressing *bZIP1* was lower than wild-type. The mean number of rosette leaves found on transformant lines 3, 5 and 8 were 0.81, 0.79 and 0.93 times fewer respectively, than the mean number found on the wild-type plants. In transformant lines 3 and 5 these differences were significant at the 99.9% and in transformant 8 at the 95% confidence level as determined by a two-tailed Student's *t*-test (fig. 4.4.5).

Plants overexpressing *bZIP1* appear to develop more slowly, producing, within seven weeks, around four-fifths the number of rosette leaves as wild-type plants. It is worth noting that the difference in rosette leaf number amongst the transformant lines of the *bZIP1* overexpressors, mirrors their pattern of *bZIP1* well (fig. 4.2.1), whereby transformants 3 and 8 have a very similar levels of expression: but with line 8 the highest and with line 5 expressing *bZIP1* at at lowest levels of the three. This pattern has been reproduced above in the number of rosette leaves (fig. 4.4.5) and suggests that levels of *bZIP1* influence the development phenotype in a dose-dependent manner.

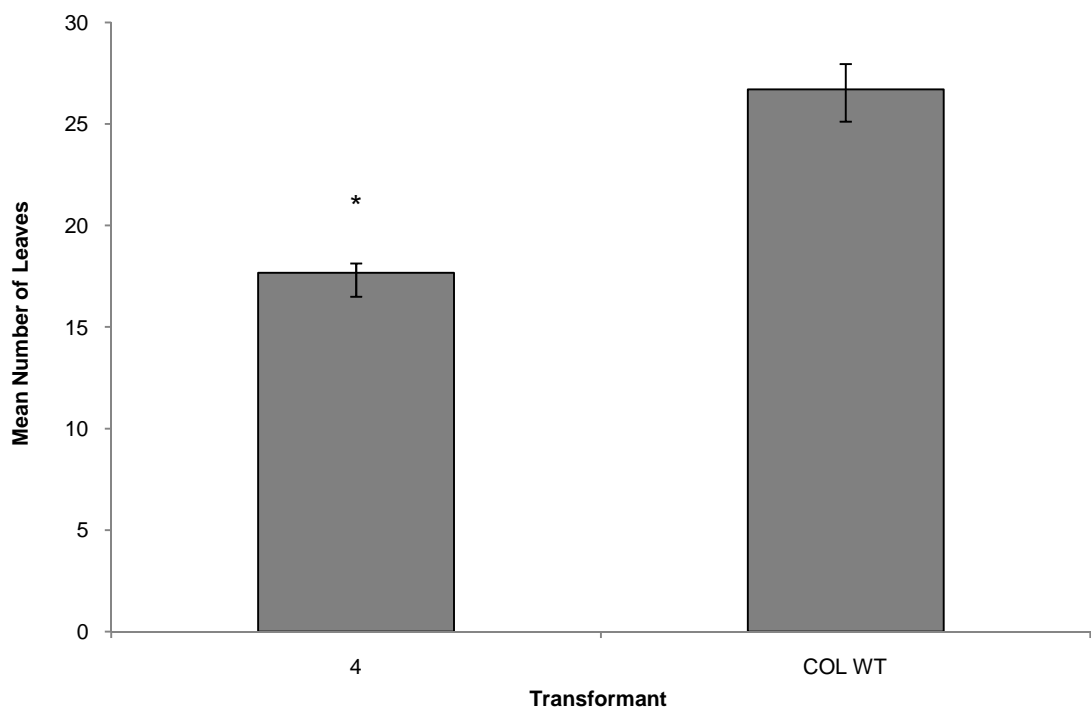


**Figure 4.4.5 Mean number of rosette leaves on 7-week-old *A. thaliana* plants overexpressing *bZIP1* compared to wild-type**

Grey bars represent the mean number of rosette leaves on short-day grown 7-week-old *A. thaliana* plants overexpressing *bZIP1* and Columbia 0 wild-type. Error bars represent the standard error of the mean. The asterisk (\*) indicates those transformant lines whose means were significantly different from Col 0 WT as determined by a two-tailed *t*-test (*t*-test results for transformant lines 3, 5 & 8 were all:  $p < 0.001$  (Transformant 8  $p < 0.05$ );  $df = 19$ ;  $t_{critical} = 2.1$ ).

After seven weeks grown under short day conditions, the mean number of rosette leaves of all the *A. thaliana* plants of the single transformant line overexpressing *CBF1* was lower than wild-type. The mean number of rosette leaves found on transformant line 4 was 0.66 times fewer than the mean number found on the wild-type plants. This difference was significant at the 99.9% confidence level as determined by a two-tailed Student's *t*-test (fig. 4.4.6).

Plants overexpressing *CBF1* appear to develop more slowly, producing, within seven weeks, just under two-thirds the number of rosette leaves as wild-type plants.

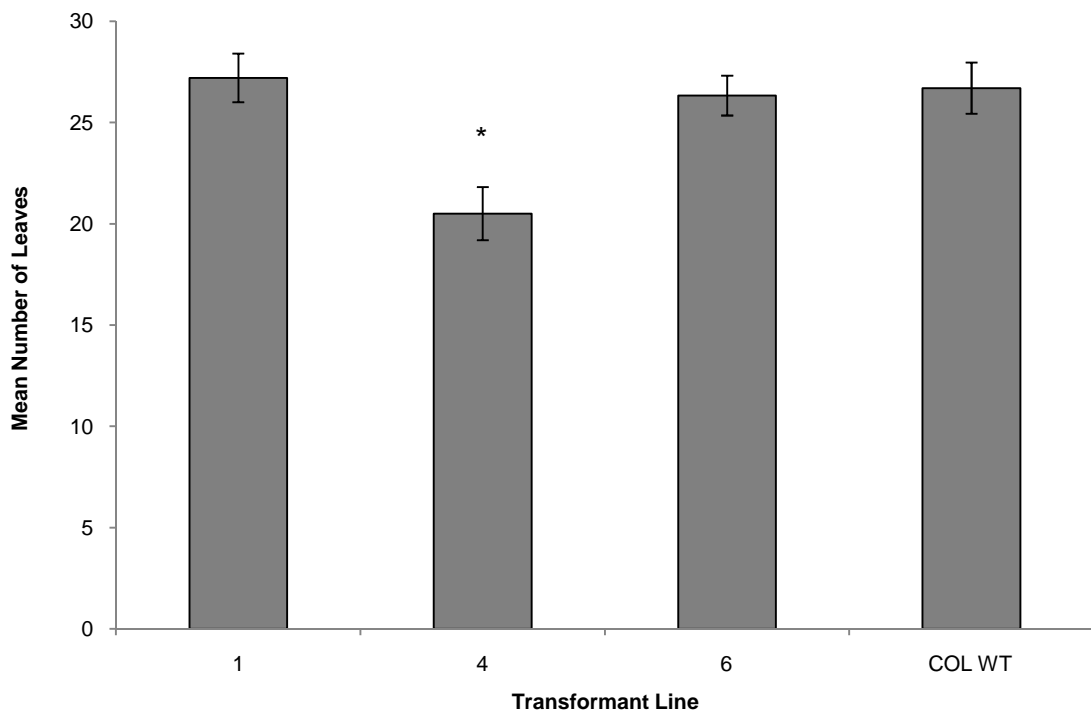


**Figure 4.4.6 Mean number of rosette leaves on 7-week-old *A. thaliana* plants overexpressing *CBF1* compared to wild-type**

Grey bars represent the mean number of rosette leaves on short-day grown 7-week-old *A. thaliana* plants overexpressing *CBF1* and Columbia 0 wild-type. Error bars represent the standard error of the mean. The asterisk (\*) indicates that plants of the single transformant line had means significantly different from Col 0 WT as determined by a two-tailed *t*-test (*t*-test result for transformant line 4 was:  $p < 0.001$ ;  $df = 18$ ;  $t_{critical} = 2.2$ )

After seven weeks grown under short day conditions, the mean number of rosette leaves of all the transformant lines of *A. thaliana* overexpressing *GATA21* was comparable to wild-type. The mean number of rosette leaves found on transformant lines 1, 4 and 6 were 1.01, 0.77 and 0.99 times fewer respectively, than the mean number found on the wild-type plants. In transformant lines 4 the difference was significant at the 99.9% confidence level as determined by a two-tailed Student's *t*-test (fig. 4.4.7).

Given that after seven weeks only one out of three of the transformant lines overexpressing *GATA21* had mean number of rosette leaves outside the distribution of the mean of wild-type, with the others lying within, it is reasonable to deduce that plants overexpressing *GATA21* do not differ from wild-type in terms of their developmental progress.

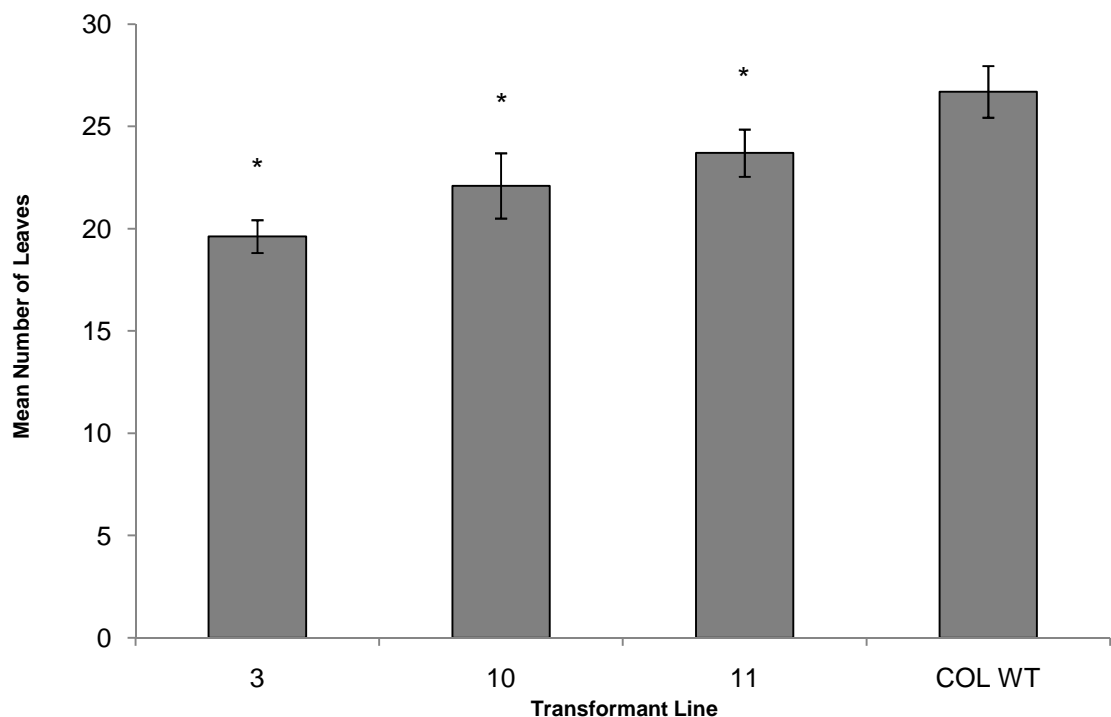


**Figure 4.4.7 Mean number of rosette leaves on 7-week-old *A. thaliana* plants overexpressing *GATA21* compared to wild-type**

Grey bars represent the mean number of rosette leaves on short-day grown 7-week-old *A. thaliana* plants overexpressing *GATA21* and Columbia 0 wild-type. Error bars represent the standard error of the mean. The asterisk (\*) indicates those transformant lines whose means were significantly different from Col 0 WT as determined by a two-tailed *t*-test (*t*-test results for transformant lines 4 was:  $p < 0.001$ ;  $df = 19$ ;  $t_{critical} = 2.1$ ).

After seven weeks grown under short day conditions, the mean number of rosette leaves of all the transformant lines of *A. thaliana* overexpressing *SRMYB* was lower than wild-type. The mean number of rosette leaves found on transformant lines 3, 10 and 11 were 0.73, 0.82 and 0.89 times fewer respectively, than the mean number found on the wild-type plants. In all transformant lines these differences were significant at the 99.9% and in transformant 11 at the 95% confidence level as determined by a two-tailed Student's *t*-test (fig. 4.4.8).

Plants overexpressing *SRMYB* appear to develop more slowly, producing, within seven weeks, around four-fifths the number of rosette leaves as wild-type plants.



**Figure 4.4.8 Mean number of rosette leaves on 7-week-old *A. thaliana* plants overexpressing *SRMYB* compared to wild-type**

Grey bars represent the mean number of rosette leaves on short-day grown 7-week-old *A. thaliana* plants overexpressing *SRMYB* and Columbia 0 wild-type. Error bars represent the standard error of the mean. The asterisk (\*) indicates those transformant lines whose means were significantly different from Col 0 WT as determined by a two-tailed *t*-test (*t*-test results for transformant lines 3, 5 & 8 were all:  $p < 0.001$  (Transformant DARK L9  $p < 0.05$ );  $df = 19$  (Transformant L3  $df = 17$ );  $t$  critical = 2.1).

## Results Summary

Plants overexpressing *bZIP1* had a substantial reduction in their aerial fresh weight after seven weeks of growth compared to wild-type (fig. 4.4.1), however the number of rosette leaves produced, although significantly different from wild-type, was only reduced by a fifth of wild-type (fig. 4.4.5). This would suggest that the overexpression of *bZIP1* has implications on the production of aerial biomass, but little or no effect on development. Considering the plants overexpressing *CBF1*, there was a dramatic reduction in the aerial fresh weight: less than a fifth of that produced by the seven week wild-type plants (fig. 4.4.2). The number of rosette leaves found on *CBF1* overexpressing plants was similarly affected, although not as severely, with roughly a third fewer leaves compared with wild-type (fig. 4.4.6). The combination of these effects suggests the overexpression of *CBF1* has profound deleterious effects on growth and development. Plants overexpressing *GATA21* were found to be around a third lighter in their aerial fresh weight compared with wild-type plants (fig. 4.4.3), but with very similar number of rosette leaves as wild-type after seven weeks growth (fig. 4.4.7). Considering both these results, it is difficult to identify any strong effects associated with the overexpression of *GATA21*. A reduction by about a third was seen in plants overexpressing *SRMYB* compared to wild-type (fig. 4.4.4), a smaller reduction of around a fifth was seen in the number of rosettes found on the same plants compared with wild-type (fig. 4.4.8). Given that both these reductions were of similar magnitude, it could be suggested that the overexpression of *SRMYB* reduced overall rate of growth and development.

The patterns described above, can be roughly identified from the photographs, particularly those with the greatest fresh weight differences such as *CBF1* (fig. 4.4.9). There are some more qualitative remarks which can be made regarding the phenotypes of the overexpressing lines which underwent seven weeks under short-day conditions. *bZIP1* overexpressors, especially lines 3 and 5 (fig. 4.4.9: A & B), had 'spoon-shaped' (Ichihashi et al., 2010) leaves when compared to wild-type (fig. 4.4.9: N), characterised by an elongated petiole with a circular leaf, whereas wild-type had a more elliptical leaf which started more proximally up the petiole. *CBF1* overexpressors (fig. 4.4.9: D) appeared very stunted in their growth and vertical height compared to wild-type, with darker leaves to wild-type but with normal morphology, which accords with observations made about *CBF1* overexpressors in the literature (Gilmour et al., 2004). Plants overexpressing *GATA21* did not exhibit any phenotypes that distinguished them

from wild-type. *SRMYB* overexpressing plants (fig. 4.4.9: H – J) demonstrated a slightly elongated petiole, but not as extreme as *bZIP1*.



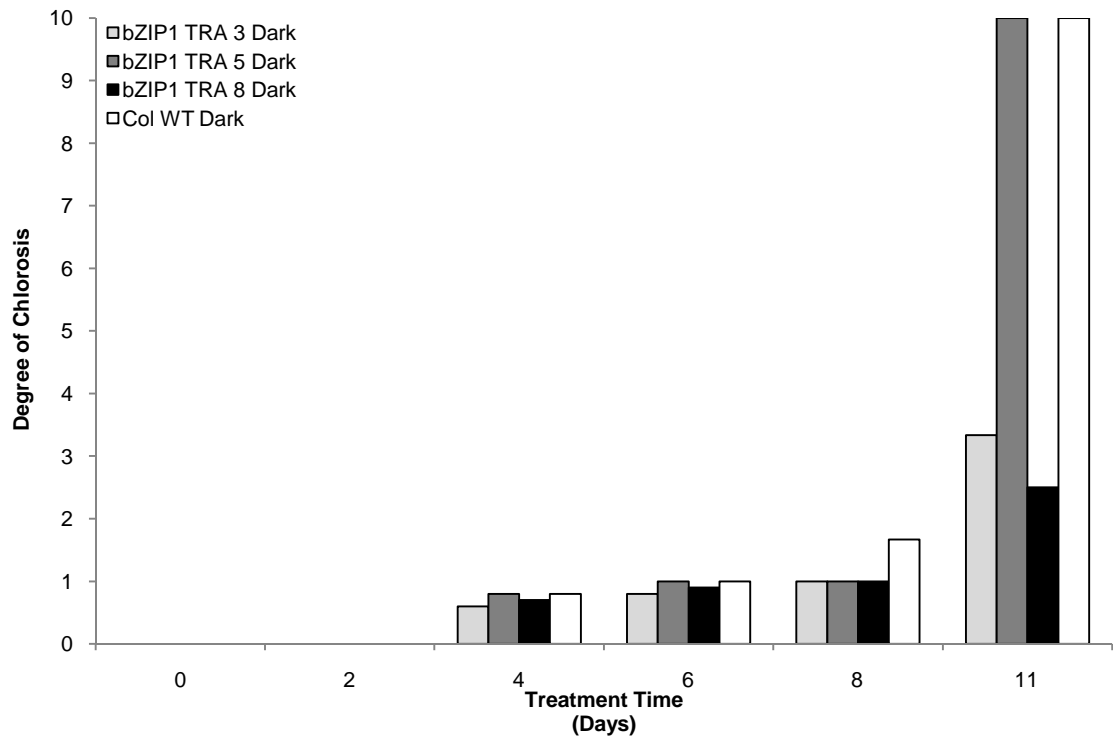
**Figure 4.4.9 Photographs of 7-week-old *A. thaliana* plants overexpressing transcription factor genes grown under short-day conditions**

Photographs of a single, seven week old representative *A. thaliana* plant from each overexpressing line ground under short day conditions (8h:16h;light/dark) at 20°C. *bZIP1* 3, 5 & 8: A – C; *CBF1*: D; *GATA21* 1, 4 & 6: E – G; *SRMYB* 3, 10 *DARKL9*: H – J; Col 0 WT: K. Bar = 40mm. Photographs taken with a Nikon CoolPix 4500.

### Results of prolonged darkness treatment

The expression of *DIN3* has been shown to be induced by periods of prolonged darkness (Fujiki et al., 2000) (§3.5/6). Conditions such as those used in the experiments of this project cause plants to experience sugar starvation, since at the end of the night-period, plants possess only a small amount of starch (Gibon et al., 2004) – most has been turned over during the night to support respiration. It is therefore instructive to consider the effects which the overexpression of transcription factors, implicated in light/dark response, have on the adaptation to even longer periods of darkness which are likely to start the processes of starvation-induced autophagy and/or senescence (Hanaoka et al., 2002).

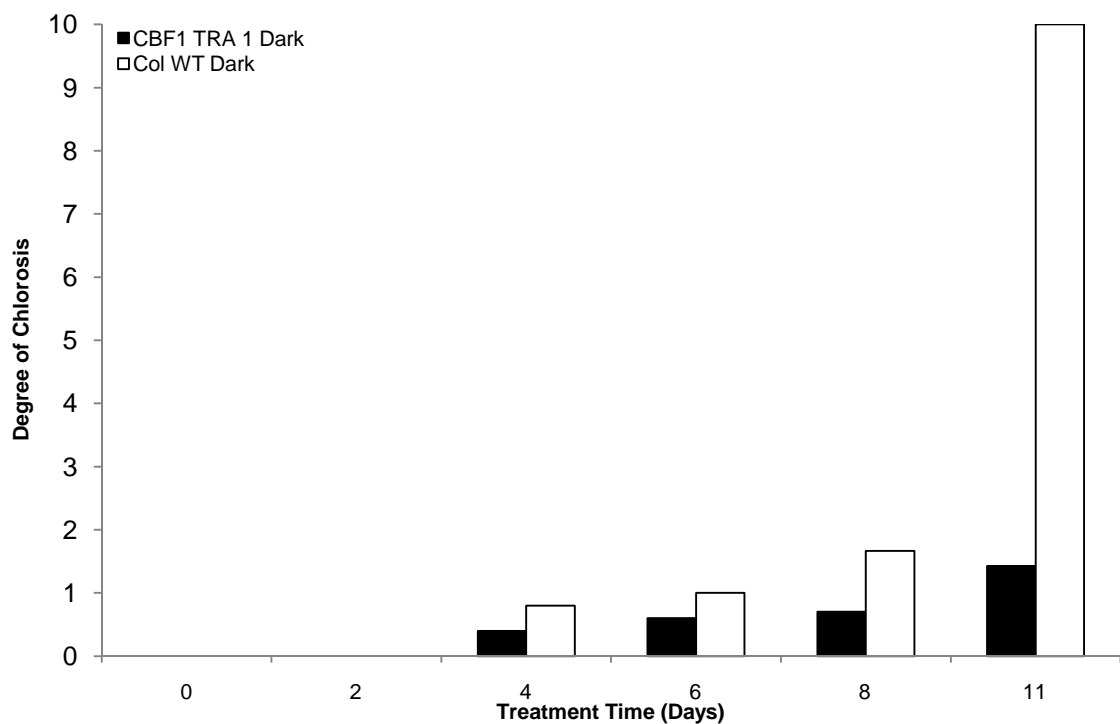
The onset of chlorosis appeared to be the same for all the overexpressing lines of *bZIP1* and wild-type, occurring at most after four days in complete darkness at 20°C. Chlorosis progressed slowly between the fourth and eighth days, with the degree of chlorosis similar amongst both the overexpressing lines and wild-type. However transformant line 5 overexpressors and wild-type plants generally yellowed more rapidly. By 11<sup>th</sup> day, the wild-type plants reached the maximum level of chlorosis: 10; characterised by having not a single green leaf on any of the plants. A large increase in the extent of chlorosis was delayed until after the eighth day in the overexpressing lines, with transformant line 5 reaching the maximum degree of senescence followed by line 3 at third less and line 8 a quarter (ratio) of the degree of chlorosis exhibited by transformant line 5 and wild-type. Amongst the control plants (kept under long-days), the chlorosis factor of *bZIP1* overexpressors 3, 5 and 8 was 0.1, 0.9 and 0.8 respectively; for wild-type this was 0.3. Two out of three of the transformant lines overexpressing *bZIP1* included plants whose degree of chlorosis was considerably lower than wild-type (fig. 4.4.10). All three transformant lines expressed *bZIP1* at levels far exceeding wild-type, however lines 3 and 8 expressed *bZIP1* at greater levels than line 5 (fig. 4.2.1).



**Figure 4.4.10 Observed chlorosis in *A. thaliana* plants of transformant lines overexpressing *bZIP1* treated with complete darkness at 20°C for 11 days compared to wild-type**

Ten 14-day-old *A. thaliana* plants of independently transformed lines 3 (light grey), 5 (mid grey) and 8 (black) overexpressing *bZIP1* and wild-type (white bars), grown under long day conditions (16h:8h/light:dark) at 20°C were then moved to complete darkness at 20°C. The observed development of chlorosis is summarised in this figure as 'Degree of Chlorosis' which is the ratio of number of plants (out of 10) which had  $\geq 1$  yellow leaves to the number which had  $\geq 1$  green leaves. These data were extracted from photographs taken at the time intervals indicated.

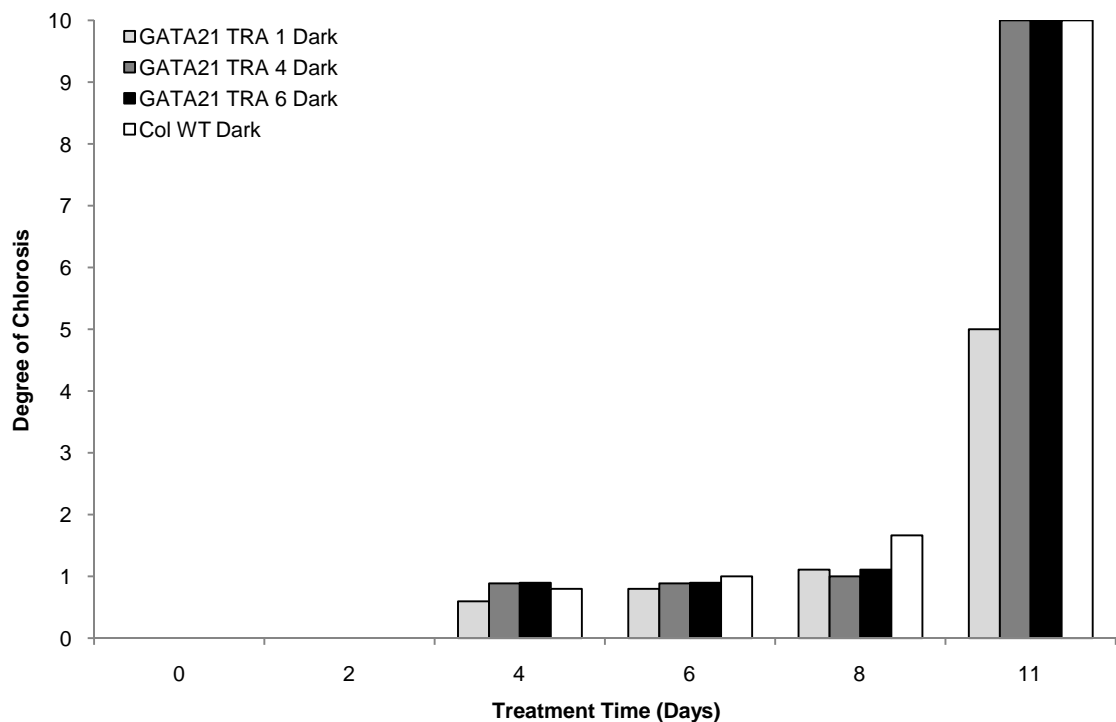
The onset of chlorosis appeared to be the same for all the overexpressing lines of *CBF1* and wild-type, occurring at most after four days in complete darkness at 20°C. Chlorosis progressed slowly between the fourth and eighth days, with the degree of chlorosis of the plants overexpressing *CBF1* usually exhibited half that of wild-type. By 11<sup>th</sup> day, the wild-type plants reached the maximum level of chlorosis: 10. With the *CBF1* overexpressing plants the increase in the degree of chlorosis increase between the eighth and 11<sup>th</sup> days of the dark treatment so that by the end, the degree of chlorosis was 1.4 as opposed to 10 in the wild-type plants (fig. 4.4.11). Amongst the control plants (kept under long-days), the degree of chlorosis in the plants overexpressing *CBF1* was 0 compared with 0.3 for wild-type. Onset of chlorosis is much delayed in plants overexpressing *CBF1* compared with wild-type.



**Figure 4.4.11 Observed chlorosis in *A. thaliana* plants of transformant lines overexpressing *CBF1* treated with complete darkness at 20°C for 11 days compared to wild-type**

Ten 14-day-old *A. thaliana* plants of independently transformed lines overexpressing *CBF1* (black bars) and wild-type (white bars), grown under long day conditions (16h:8h/light:dark) at 20°C were then moved to complete darkness at 20°C. The observed development of chlorosis is summarised in this figure as 'Degree of Chlorosis' which is the ratio of number of plants (out of 10) which had  $\geq 1$  yellow leaves to the number which had  $\geq 1$  green leaves. These data were extracted from photographs taken at the time intervals indicated.

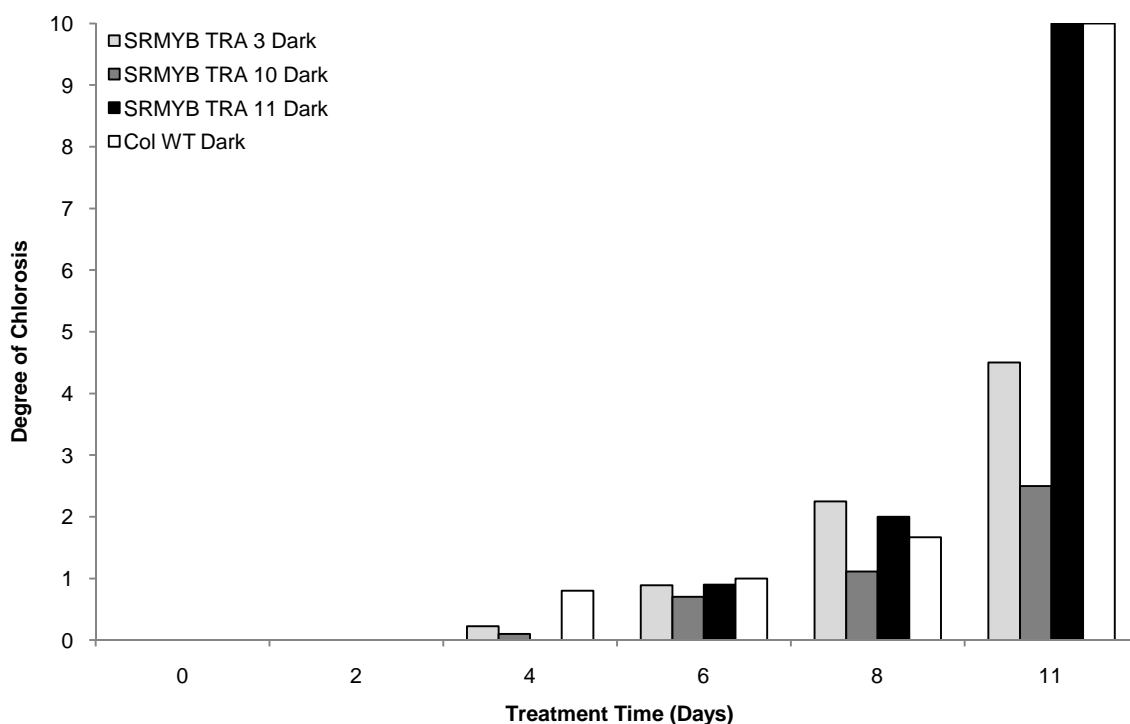
The onset of chlorosis appeared to be the same for all the overexpressing lines of *GATA21* and wild-type, occurring at most after four days in complete darkness at 20°C. Chlorosis progressed appreciably in the same manner amongst both the overexpressing lines and wild-type. By the 11<sup>th</sup> day wild-type plants had reached the maximum level of chlorosis: 10 as well as two out of three of the overexpressing lines (4 and 6) and line 1 half that extent. Amongst the control plants (kept under long-days), the degree of chlorosis of *GATA21* overexpressors 1, 4 and 6 was 0.2, 0.4 and 0 respectively; for wild-type this was 0.3. Overall the transformant lines overexpressing *GATA21* exhibited no appreciable difference in the progress of chlorosis and two out of three of the lines reached the maximum degree of chlorosis by the 11<sup>th</sup> day matching that of wild-type (fig. 4.4.12).



**Figure 4.4.12 Observed chlorosis in *A. thaliana* plants of transformant lines overexpressing *GATA21* treated with complete darkness at 20°C for 11 days compared to wild-type**

Ten 14-day-old *A. thaliana* plants of independently transformed lines 1 (light grey), 4 (mid grey) and 6 (black) overexpressing *GATA21* and wild-type (white bars), grown under long day conditions (16h:8h/light:dark) at 20°C were then moved to complete darkness at 20°C. The observed development of chlorosis is summarised in this figure as 'Degree of Chlorosis' which is the ratio of number of plants (out of 10) which had  $\geq 1$  yellow leaves to the number which had  $\geq 1$  green leaves. These data were extracted from photographs taken at the time intervals indicated.

The onset of chlorosis occurred at different times for the overexpressing lines of *SRMYB* and wild-type, with wild-type and lines 3 and 10 occurring at most after four and line 11 after six days in complete darkness at 20°C. Until the eighth day of the treatment wild-type the progression of chlorosis amongst the wild-type plants was more advanced. There were some signs of chlorosis amongst lines 3 and 10 on the fourth day, however it was not until the sixth day that their degree of chlorosis become more similar but still slightly lower than wild-type. By the sixth day line 11 had also caught up with the other transformant lines overexpressing *SRMYB*. On the eighth day, the extent of chlorosis in plants of *SRMYB* lines 3 and 11 had progressed beyond wild-type and line 10. Following another three days in complete darkness the wild-type plants had reached the maximum level of chlorosis, matched by line 11. *SRMYB* lines 3 and 10 exhibited just under a half and a quarter the levels of chlorosis seen in either the line 11 or wild-type plants. Two out of three transformant lines overexpressing *SRMYB*, had plants whose degree of chlorosis was considerably lower than wild-type (fig. 4.4.13). Comparing the final extent of chlorosis between *SRMYB* lines 3 and 10 and their relative *SRMYB* expression levels (fig. 4.2.5), it is reasonable to suggest that the greater extent of chlorosis in line 3 is due to its higher expression of *SRMYB* compared to line 10. *SRMYB* overexpressing line 11 was produced previously using a different 35S expression vector to the other two *SRMYB* overexpression lines. A Northern blot found very high levels of *SRMYB* expression in this line (data not shown). However its expression was not compared with the other *SRMYB* lines via qRT-PCR. Along with the mean fresh weight (fig. 4.4.4) this result emphasises that the level of *SRMYB* transcript has a dramatic effect on growth and development as well as the response to starvation-induced chlorosis. However since there is no one *SRMYB* overexpressor phenotype, especially in terms of autophagy /senescence, it is not merely the absolute levels of *SRMYB* transcript which mediate this effect, since all lines expressed *SRMYB* at many times the levels found in wild-type plants. Instead the different levels of *SRMYB* seen in the three overexpressors must cause a different physiological response.



**Figure 4.4.13 Observed chlorosis in *A. thaliana* plants of transformant lines overexpressing *SRMYB* treated with complete darkness at 20°C for 11 days compared to wild-type**

Ten 14-day-old *A. thaliana* plants of independently transformed lines 3 (light grey), 10 (mid grey) and 11 (black) overexpressing *SRMYB* and wild-type (white bars), grown under long day conditions (16h:8h/light:dark) at 20°C were then moved to complete darkness at 20°C. The observed development of chlorosis is summarised in this figure as 'Degree of Chlorosis' which is the ratio of number of plants (out of 10) which had  $\geq 1$  yellow leaves to the number which had  $\geq 1$  green leaves. These data were extracted from photographs taken at the time intervals indicated.

### Results Summary

The starvation-induced chlorosis patterns observed in the overexpressing lines can be divided into two principal groups. The first are those plants which have a broadly similar pattern to wild-type and included *GATA21* overexpressors. These had two out of three of their overexpressing lines with the same degree of chlorosis at the end of the 11 day dark treatment. These two lines did have chlorosis profiles which different appeared different to wild-type in the progression to the final stages of chlorosis (fig. 4.4.12). The second group contain those plants, the majority of which had a considerably lower degree of chlorosis at the end of the 11 day dark treatment compared to wild-type and included plants overexpressing *bZIP1*, *CBF1* and *SRMYB* (figs. 4.4.10/11/13). All of these lines had two out of three of their transformant lines with degrees of chlorosis at least less than half of that of wild-type. In all cases the chlorosis profiles were different to wild-type: *bZIP1* took longer to enter into the latter stages of chlorosis (fig. 4.4.10),

*CBF1* never reached the latter stages within the 11 days (fig. 4.4.11) and two out of three of the *SRMYB* lines progressed and consistently slower rate to wild-type (fig. 4.4.13).

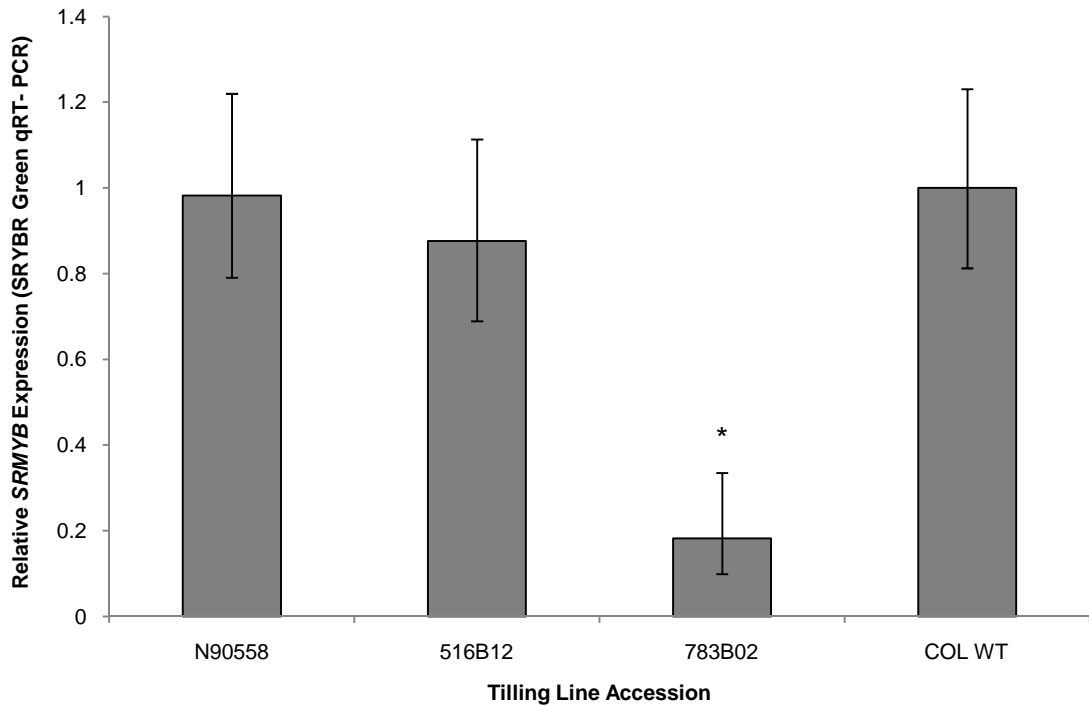
## 4.5 Comparison of *SRMYB* expression between mutants and Col 0 WT

In addition to using seedlings overexpressing *SRMYB* to investigate the effect of the *SRMYB* transcription factor on *DIN3* expression and growth and development, seedlings with lesions caused by point mutations or T-DNA insertions in their *SRMYB* gene were tested for the effect of *SRMYB* at the other end of its expression range. In designing experiments using these tilling lines there was the assumption that the tilling lines would have reduced *SRMYB* expression compared to wild-type. For sound conclusions to be drawn from subsequent experiments using the tilling lines, this assumption would have to be proved correct.

The tilling line accessions N90558, 516B12, 783B02 came through a preliminary screen, using semi-quantitative PCR using full length cDNA, as having potentially altered *SRMYB* expression levels compared to wild-type. A more definitive analysis was required and the results of the qRT-PCR analysis are considered presently.

The qRT-PCR analysis of *SRMYB* expression carried out on the *SRMYB* tilling lines was relative to the *SRMYB* expression found in Col 0 WT. All the tilling lines demonstrate altered *SRMYB* expression compared with to wild-type expression levels. In lines N90558 and 516B12 *SRMYB* expression is 0.98 and 0.88 times that of wild-type respectively and was found not to be significantly different from wild-type expression in this analysis. Line 783B02 however, demonstrated dramatically lower expression: 0.18 times that of wild-type expression which was found to be significantly different at the 95% confidence level (fig. 4.5.1).

This analysis shows that the lesions to the *SRMYB* gene possessed by the tilling lines did in fact alter *SRMYB* expression compared with wild-type. Whilst only in one accession, 783B02, was expression significantly reduced compared with wild-type, both the other accessions were lower in recorded *SRMYB* expression. Crucially for the purposes of the latter experiments, all the tillings were confirmed not to express *SRMYB* at greater levels than wild-type.



**Figure 4.5.1 *SRMYB* expression amongst 7-day-old seedlings of *SRMYB* tilling lines relative to Col 0 WT expression levels.**

Relative *SRMYB* expression levels in 7-day-old seedlings of *SRMYB* mutant lines (standardised against *PEX4* expression) and Col 0 WT grown under long day (16h/8h light/dark) at 20°C. Expression levels are relative to the Col 0 WT sample. The asterisk (\*) indicates those data sets which were outside the distribution of the Col 0 WT data set. Error bars represent the Student's *t*-distribution maxima and minima; df=4.

## 4.6 Analysis of *DIN3* expression in *SRMYB* tilling lines under various conditions

As explained in the previous section, the use of *SRMYB* tilling lines with reduced expression levels of *SRMYB* (§4.5), a transcription factor with a putative regulatory role over *DIN3*, provides additional information in the investigation of this role but at the other end of the *SRMYB* expression level range to the experiments using lines overexpressing *SRMYB*.

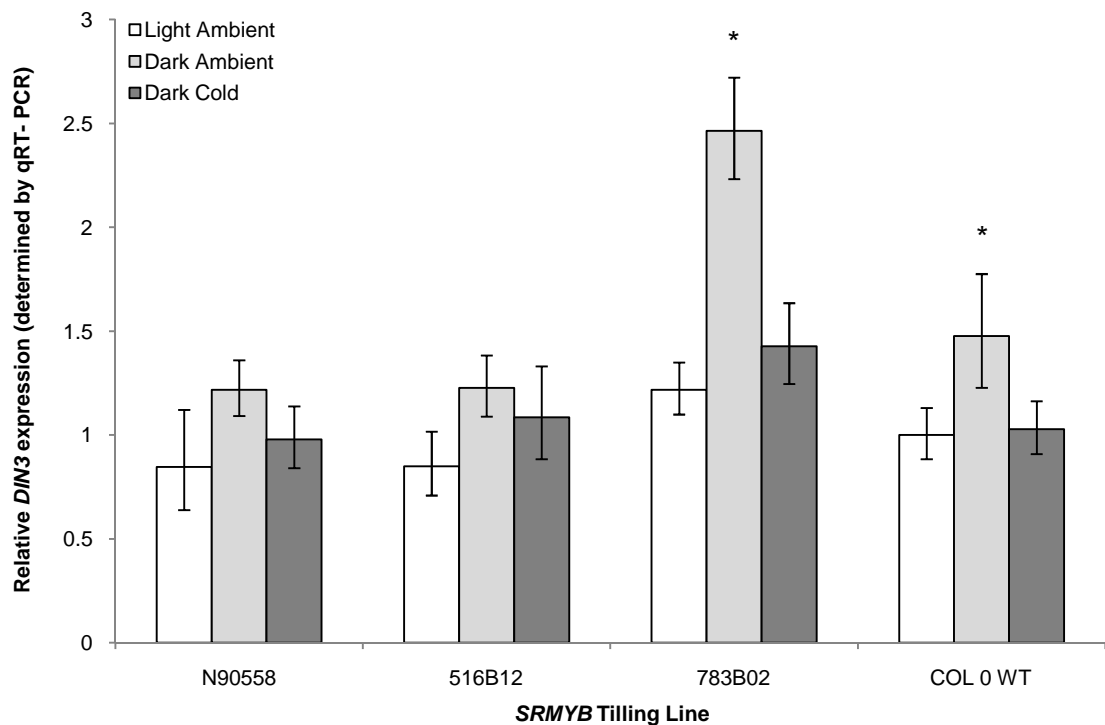
The approach of the experiment was the same, seedlings of the three tilling line accessions, N90558, 516B12 and 783B02, were grown alongside Col 0 WT to 7 days old and then treated with either three hours of light or darkness at 20°C or darkness at 5°C, in order to investigate what effect, if any, reduced levels of *SRMYB* subsequently had on the expression of *DIN3* under these environmental conditions. Expression levels of *DIN3* were analysed by qRT-PCR.

*DIN3* expression amongst the seedlings of the *SRMYB* tilling lines mirrored the characteristic expression pattern seen in the Col 0 WT: an induction in *DIN3* expression following a period of extended darkness at 20°C, with repression if carried out at 5°C. The dark-induction of *DIN3* expression was weaker than Col 0 WT in tilling lines N90558 and 516B12, both 1.2 times the *DIN3* expression seen in the light sample of Col 0 WT (constitutive). The overall magnitude of *DIN3* expression in tilling line 783B02 was greater than Col 0 WT. Three hours of darkness at 20°C elicited a strong induction in *DIN3* expression, 2.5 times that of constitutive. In all cases the dark-induced expression of *DIN3* was greater than the light sample; in the case of 783B02, as with Col 0 WT, these were significantly different.

Mirroring Col 0 WT, there was a repression of the dark-induction of *DIN3* expression following three hours of darkness at 5°C in tilling lines N90558, 516B12 and 783B02: 0.98, 1.1 and 1.4 times constitutive respectively (fig. 4.6.1).

It is also important to compare the dark-induced *DIN3* expression of the tilling lines with that of Col 0 WT. Following three hours of darkness at 20°C the *DIN3* expression of tilling lines N90558, 516B12 and 783B02 were 0.82, 0.83 and 1.66 times that of the Col 0 WT expression level. Only the 783B02 expression was found to be significant (fig. 4.6.1).

In all tilling lines the characteristic dark-induction and cold repression of *DIN3* expression was seen. This dark induction was greatest in the 783B02 seedlings, whose *DIN3* expression was significantly greater than Col 0 WT. Lines N90558 and 516B12 did not exhibit *DIN3* expression level significantly different to Col 0 WT, but were slightly lower overall. It is evident then that lesions to the *SRMYB* gene which result in a reduction of *SRMYB* expression to varying degree result in a change in *DIN3* expression levels, but not the character of the response to changes in environmental conditions.



**Figure 4.6.1 *DIN3* expression in 7-day-old *SRMYB* tilling line seedlings relative to Col 0 WT following three hour light and dark ambient and dark cold treatments.**

Relative *DIN3* expression levels in 7-day-old *SRMYB* tilling line seedlings (standardised against *PEX4* expression) seedlings after 3h ambient (20°C) light (white), dark (light grey) or dark cold (5°C) (dark grey) treatments. Expression levels are relative to the light sample of Col 0 WT. The asterisk (\*) indicates those data sets which were outside the distribution of the corresponding light ambient (20°C) data set. Error bars represent the Student's *t*-distribution maxima and minima; df=9.

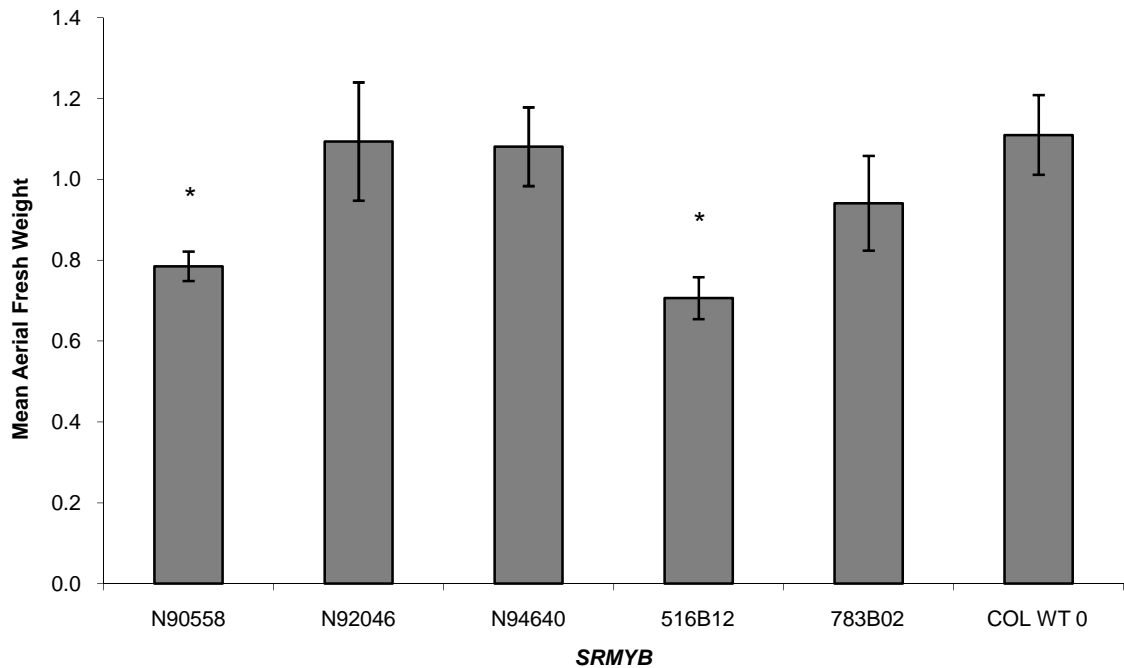
## 4.7 Growth effects of *SRMYB* tilling and T-DNA lines

Earlier work (Knight, H., unpublished) using three tilling lines and two others with T-DNA insertions within the *SRMYB* revealed novel phenotypes arising when these plants were grown under short-day conditions. With the putative role of *SRMYB* as a negative regulator of sugar state, it was considered instructive to design an experiment to investigate this. The experiment sought to achieve this by observing the phenotype and response to short-days (8h:16h/light:dark) for nine weeks and prolonged darkness at 20°C and 5°C (20 and 49 days respectively), all treatments which decrease the availability of carbon from photosynthesis to a lesser or greater degree, in MYB mutant plants compared with those of wild-type.

The short-day grown plants were analysed quantitatively by measuring their aerial fresh weight, but also by taking photographs during the last few weeks of the experiment to monitor the emergence of a specific senescing phenotype (Dong et al., 2009). The other two experiments treating the MYB line plants to either complete darkness at 5 or 20°C were photographed at day 0, 5, 7, 10, 13, 20, 24, 31, 38, 45 and 49. Latterly, a comparison was made of the ratio of the number of plants, of each line, of each treatment temperature, which had  $\geq 1$  yellow leaf to the number of plants which had  $\geq 1$  green leaf. In each case this ratio is compared alongside Col 0 wild-type plants; the wild-type data are not pair-wise comparisons rather the same wild-type data is use each time. This analysis permitted the charting of the course of the dark-induced senescence comparing the effects of both temperature and mutations to the *SRMYB* gene.

### Results of growth under short-days

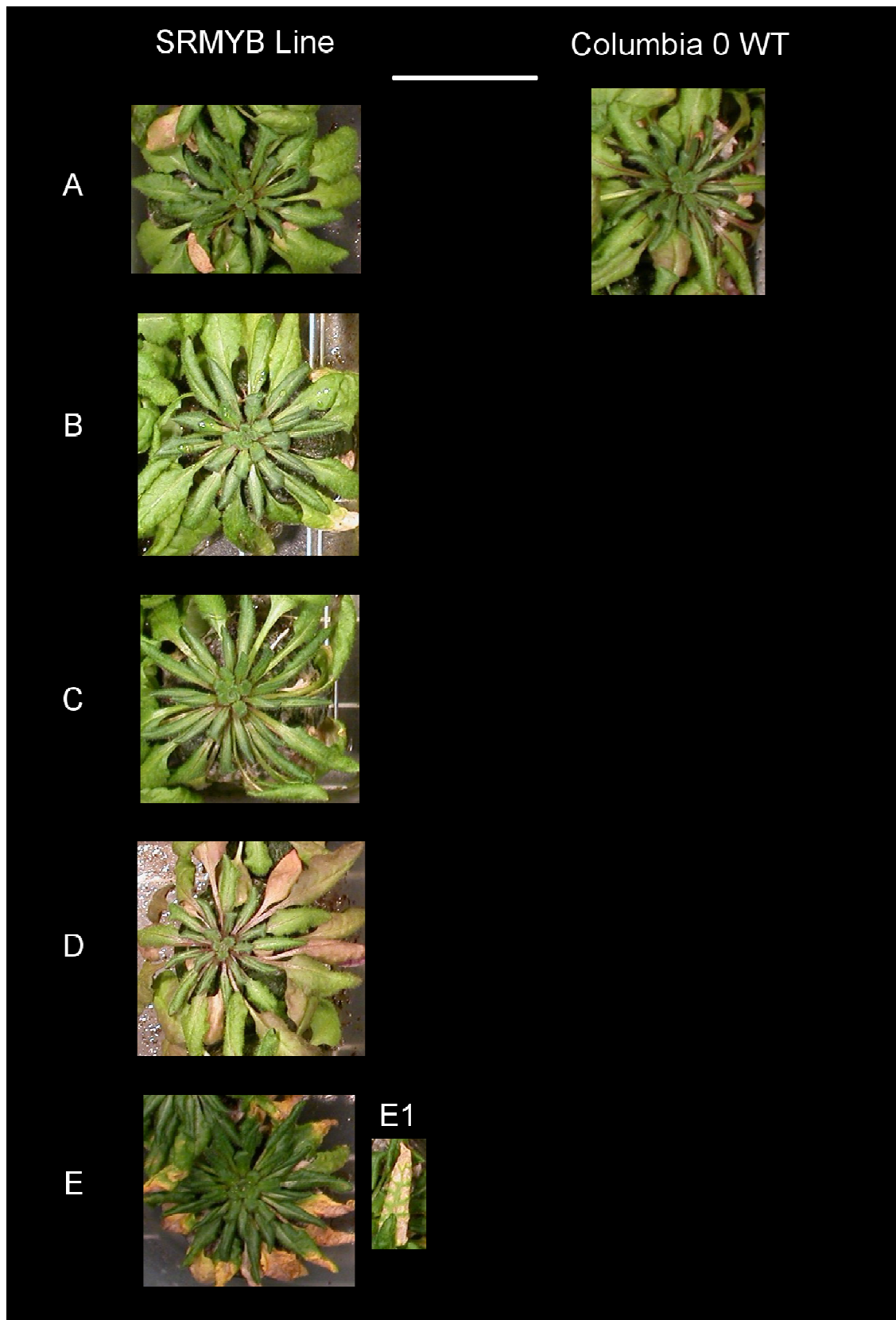
Following nine weeks growing under short-days at 20°C a variation in the mean aerial fresh weight of the tilling and T-DNA insertion lines emerged when compared to wild-type. Plants of two of the tilling lines, N92046 and N94640, were almost identical in their mean fresh weight compared to wild-type 0.98 and 0.97 times the mean fresh weight of wild-type. Plants of T-DNA insertion line 783B02 had a lower mean fresh weight compared to wild-type: 0.85 times that of wild-type (fig. 4.7.1). However a two-tailed Student's *t*-test revealed this difference not to be significant at the 95% confidence level. Plants of tilling line N90558 and T-DNA insertion line 516B12 had mean aerial fresh weights 0.7 and 0.63 times that of wild-type, both these differences were found to be significant at the 99.9% confidence level by a two-tailed Student's *t*-test.



**Figure 4.7.1 Mean aerial fresh weight of short-day grown 9-week-old *A. thaliana* plants of EMS tilling lines or T-DNA insertional *SRMYB* mutants compared to wild-type**

Grey bars represent the mean aerial fresh weight of 9-week-old *A. thaliana* plants (grown under short-days (8h:16h/light:dark) at 20°C) of EMS (tilling) lines and T-DNA insertional *SRMYB* mutants compared to wild-type. Error bars represent the standard error of the mean. The asterisk (\*) indicates those transformant lines whose means were significantly different from Col 0 WT as determined by a two-tailed *t*-test (*t*-test results for N90558 and 516B12 were both:  $p < 0.001$ ;  $df = 18$ ;  $t_{critical} = 2.2$ ).

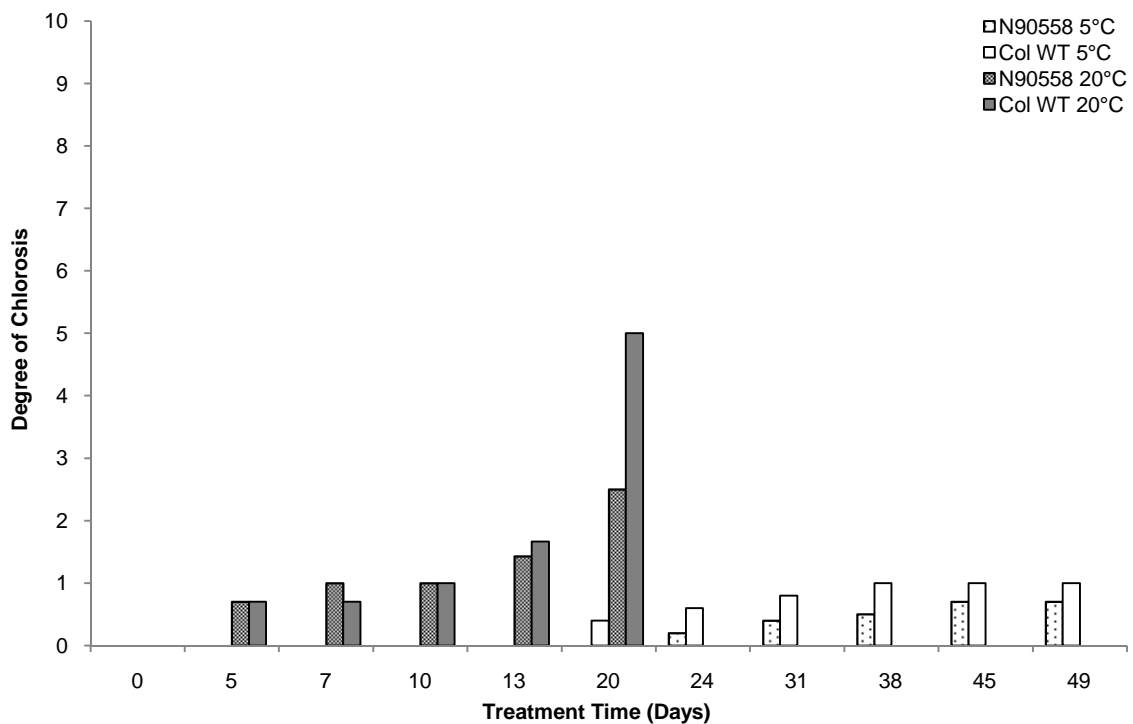
Qualitative comparisons can be made of particular phenotypes which arose after nine weeks under short-day conditions. This wild-type short-day phenotype was characterised by a darker green inner rosette with narrow in-rolling leaves, and in the outer rosette some senescing leaves, identified by a yellow (chlorosis) and/or purple (indicative of anthocyanin accumulation; (Peng et al., 2008) tint. The *SRMYB* tilling lines did not exhibit phenotypes distinguishable from wild-type during the course of the nine weeks of ground under short-day conditions (fig. 4.7.2: A – C). They did match the wild-type plants in their phenotypic response to growth in short-day conditions with senescence of their older outer rosette leaves. The *SRMYB* T-DNA lines had identifiable differences in their short-day phenotypes with 516B12 exhibiting the symptoms senescence to a greater degree and more extensive in terms of the number of leaves affected (fig. 4.7.2: D). 783B02 had extensive chlorosis amongst its old outer rosette leaf and senescence appeared to be restricted to these leaves. Notably 783B02 plants lacked any purple tint to senescing leaves, which may suggest a lack of anthocyanin accumulation. Where senescence was in progress a characteristic phenotype was seen manifested by banded yellow and green leaves (fig. 4.7.2: E1). The centre of the rosette remained a very dark green (fig. 4.7.2: E).



**Figure 4.7.2** Photographs of nine-week-old *A. thaliana* grown under short-day conditions. Photographs of a single, nine-week-old representative *A. thaliana* plant from each a *SRMYB* tilling or T-DNA line or Col 0 WT, grown under short-day conditions (8h:16h;light/dark) at 20°C. N90558: A; N92046: B; N94640: C; 516B12: D; 783B02: E; detail of 783B02 leaf senescence phenotype: E1. Bar = 40mm. Photographs taken with a Nikon CoolPix 4500.

### Results of prolonged darkness treatment

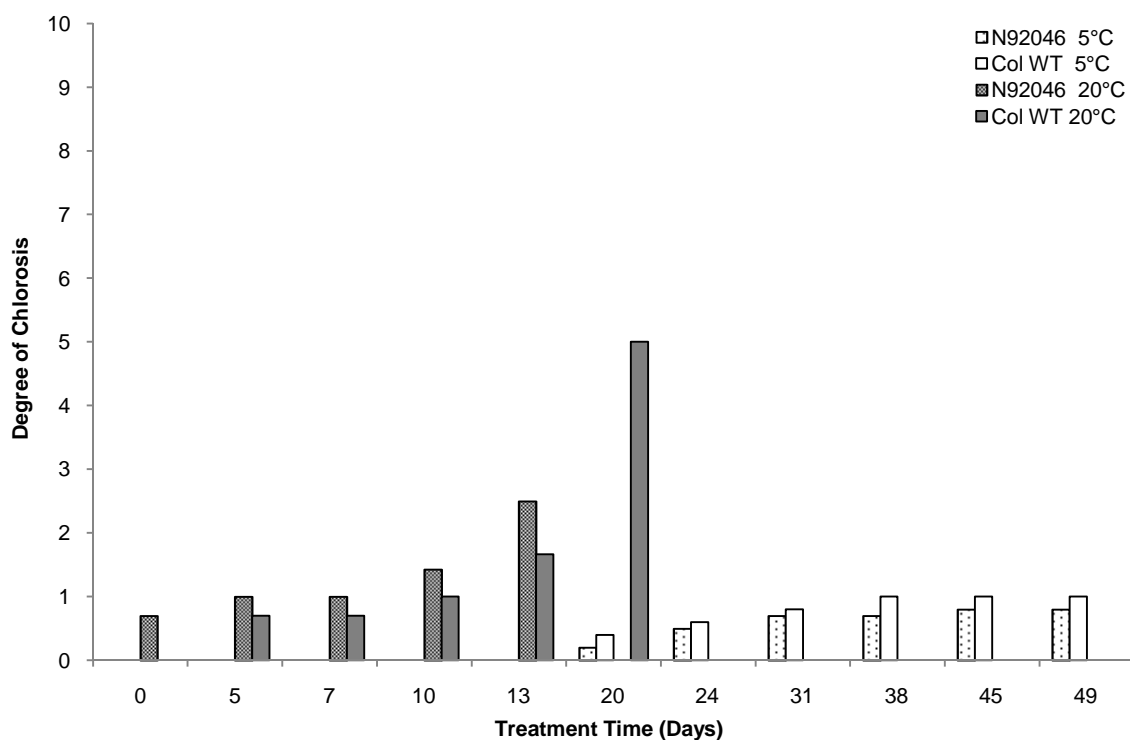
Differences are identifiable in both the onset and rate of senescence between the temperature treatments carried out on the light starved plants. In both tilling line N90558 and wild-type senescence began earlier in those plants in darkness at 20°C compared with 5°C, with onset occurring at least 15 and 19 days earlier in plants of N90558 and wild-type respectively (fig. 4.7.3). The progression of senescence at 20°C remained broadly the same up to 10 days into the dark treatment, after which senescence appeared to proceed more rapidly in the wild-type plants, and their degree of senescence was greater than those of N90558. The onset of senescence occurred earlier in the wild-type plants at 5°C and their senescence continued more rapidly compared with the N90558 plants (fig. 4.7.3).



**Figure 4.7.3 Observed chlorosis in *A. thaliana* plants of EMS tilling line N90558 treated with complete darkness at 5 and 20°C for 49 days compared to wild-type**

Ten, 16-day-old *A. thaliana* plants of tilling line N90558 (patterned bars) and wild-type (solid bars), grown under long day conditions (16h:8h/light:dark) at 20°C were then moved to complete darkness at either 5 (white bars) or 20°C (grey bars). The observed development of chlorosis is summarised in this figure as 'Degree of Chlorosis' which is the ratio of number of plants (out of 10) which had  $\geq 1$  yellow leaves to the number which had  $\geq 1$  green leaves. These data were extracted from photographs taken at the time intervals indicated.

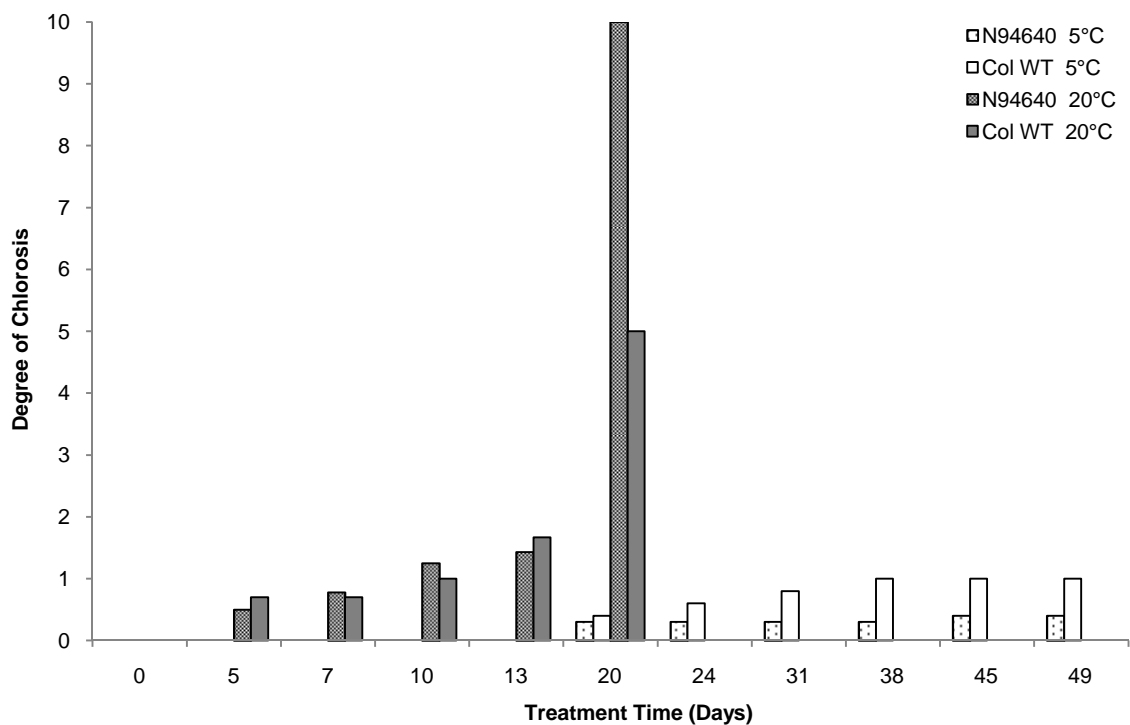
The same overall differences were identifiable in both the onset and rate of senescence between the temperature treatments carried out on these light starved plants. In both tilling line N92046 and wild-type chlorosis began earlier in those plants in darkness at 20°C compared with 5°C, with onset occurring at least 15 and 20 days earlier in wild-type and plants of N92046 respectively (fig. 4.7.4). The progression of chlorosis at 20°C appeared to proceed more rapidly in the N92046 plants, and their degree of chlorosis was greater than those of wild-type. Whilst the onset of senescence occurred simultaneously between the plant types at 5°C, the progression of chlorosis continued more rapidly in those of wild-type compared with the N90558 plants (fig. 4.7.4).



**Figure 4.7.4 Observed chlorosis in *A. thaliana* plants of tilling line N92046 treated with complete darkness at 5 and 20°C for 49 days compared to wild-type**

Ten, 16-day-old *A. thaliana* plants of tilling line N92046 (patterned bars) and wild-type (solid bars), grown under long day conditions (16h:8h/light:dark) at 20°C were then moved to complete darkness at either 5 (white bars) or 20°C (grey bars). The observed development of chlorosis is summarised in this figure as 'Degree of Chlorosis' which is the ratio of number of plants (out of 10) which had  $\geq 1$  yellow leaves to the number which had  $\geq 1$  green leaves. These data were extracted from photographs taken at the time intervals indicated.

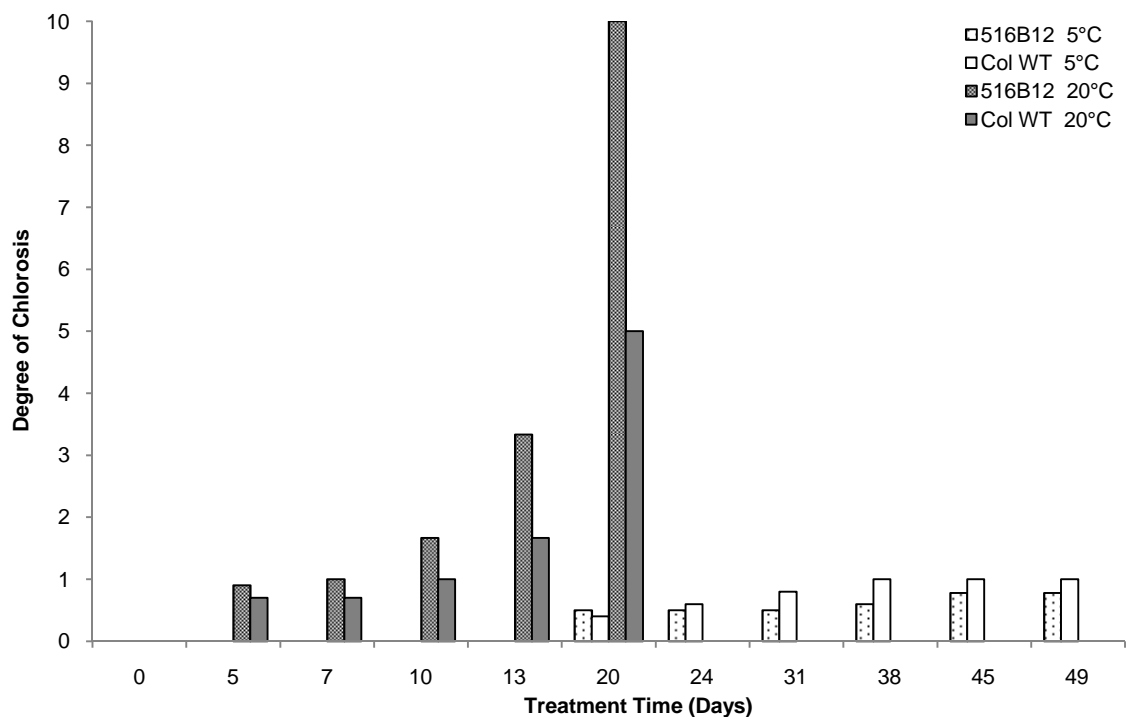
The same overall differences were identifiable in both the onset and progression of chlorosis between the temperature treatments carried out on these light starved plants. In both tilling line N94640 and wild-type chlorosis began earlier in those plants in darkness at 20°C compared with 5°C, with onset occurring at least 15 days earlier in both types plants (fig. 4.7.5). The progression of chlorosis at 20°C at first appeared to proceed more rapidly in the wild-type plants. However N94640 caught up and proceeded at broadly the same pace until overtaking with twice the degree of chlorosis compared to wild-type. Whilst the onset of chlorosis occurred simultaneously between the plant types at 5°C, the progression of chlorosis continued more rapidly in those of wild-type compared with the N94640 plants, where it seemed to progress very slowly after 20 days and never reaching the same extent as wild-type (fig. 4.7.5).



**Figure 4.7.5 Observed chlorosis in *A. thaliana* plants of tilling line N94640 treated with complete darkness at 5 and 20°C for 49 days compared to wild-type**

Ten, 16-day-old *A. thaliana* plants of tilling line N94640 (patterned bars) and wild-type (solid bars), grown under long day conditions (16h:8h/light:dark) at 20°C were then moved to complete darkness at either 5 (white bars) or 20°C (grey bars). The observed development of chlorosis is summarised in this figure as 'Degree of Chlorosis' which is the ratio of number of plants (out of 10) which had  $\geq 1$  yellow leaves to the number which had  $\geq 1$  green leaves. These data were extracted from photographs taken at the time intervals indicated.

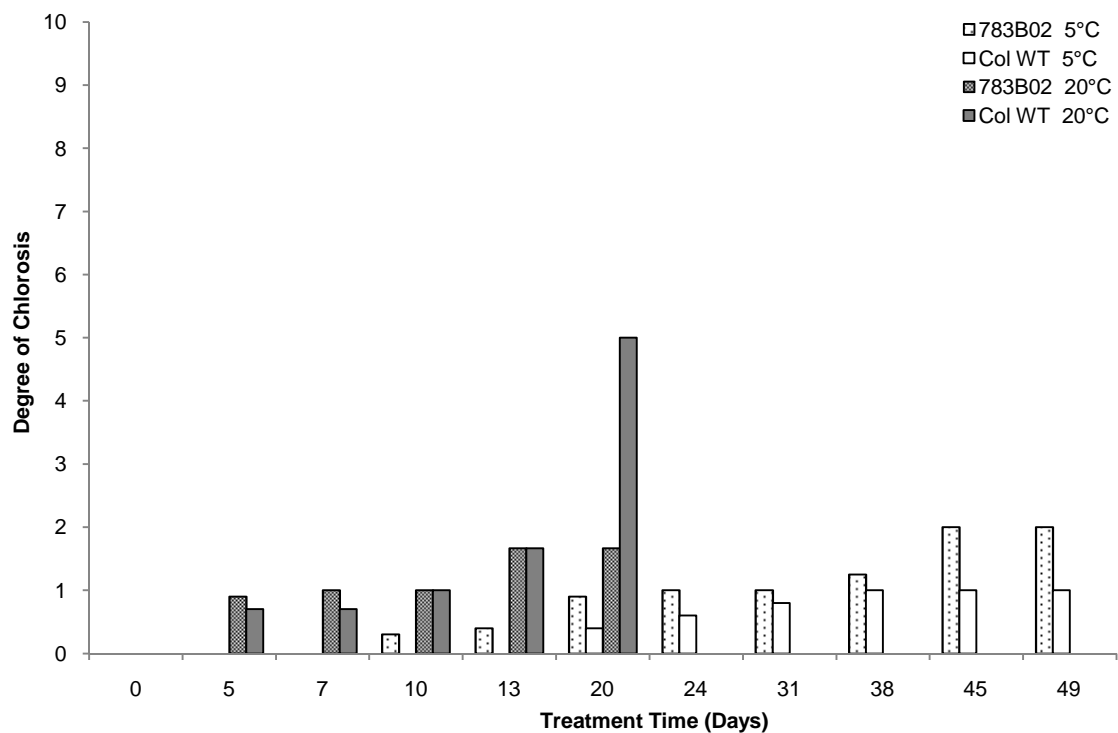
The same overall differences were identifiable in both the onset and progression of chlorosis between the temperature treatments carried out on these light starved plants. In both T-DNA line 516B12 and wild-type chlorosis began earlier in those plants in darkness at 20°C compared with 5°C, with onset occurring at least 15 days earlier in both types plants (fig. 4.4.6). The progression of chlorosis at 20°C appeared to proceed much more rapidly in the 516B12 plants, and their degree of chlorosis was twice that of wild-type. The progression of chlorosis at 5°C was broadly the same between the plant types, however wild-type overtook the 516B12 plants after the 24<sup>th</sup> day, and chlorosis in 516B12 plants progressed more slowly never reached the same degree as wild-type (fig. 4.7.6).



**Figure 4.7.6 Observed chlorosis in *A. thaliana* plants of T-DNA line 516B12 treated with complete darkness at 5 and 20°C for 49 days compared to wild-type**

Ten, 16-day-old *A. thaliana* plants of T-DNA line 516B12 (patterned bars) and wild-type (solid bars), grown under long day conditions (16h:8h/light:dark) at 20°C were then moved to complete darkness at either 5 (white bars) or 20°C (grey bars). The observed development of chlorosis is summarised in this figure as 'Degree of Chlorosis' which is the ratio of number of plants (out of 10) which had  $\geq 1$  yellow leaves to the number which had  $\geq 1$  green leaves. These data were extracted from photographs taken at the time intervals indicated.

The same overall differences were identifiable in both the onset and progression of chlorosis between the temperature treatments carried out on these light starved plants. In both T-DNA line 783B02 and wild-type chlorosis began earlier in those plants in darkness at 20°C compared with 5°C, with onset occurring at least 5 and 19 days earlier in plants of 783B02 and wild-type respectively (fig. 4.7.7). The progression of chlorosis at 20°C at first appeared to proceed more rapidly in the 783B02 plants, however wild-type caught up around the 10<sup>th</sup> day and proceeded at broadly the same pace until overtaking with more than eight times the degree of chlorosis compared to 783B02. The onset of chlorosis occurred much earlier in the 783B02 plant types at 5°C, which began senescing at least 10 days before wild-type. The progression of chlorosis in the 783B02 plants continued very much more rapidly compared with those of wild-type, and also reached at least twice the degree of senescence (fig. 4.7.7).



**Figure 4.7.7 Observed chlorosis in *A. thaliana* plants of T-DNA line 783B02 treated with complete darkness at 5 and 20°C for 49 days compared to wild-type**

Ten, 16-day-old *A. thaliana* plants of T-DNA line 783B02 (patterned bars) and wild-type (solid bars), grown under long day conditions (16h:8h/light:dark) at 20°C were then moved to complete darkness at either 5 (white bars) or 20°C (grey bars). The observed development of chlorosis is summarised in this figure as 'Degree of Chlorosis' which is the ratio of number of plants (out of 10) which had  $\geq 1$  yellow leaves to the number which had  $\geq 1$  green leaves. These data were extracted from photographs taken at the time intervals indicated.

## Results Summary

The effect of growth under short-days was analysed by mean aerial fresh weight, from this three groups within the EMS tilling and T-DNA insertional mutant lines emerged. The first includes lines N90558 and 516B12 which had significantly smaller mean aerial fresh weights compared to wild-type. The second includes 783B02 alone, which although having a mean fresh weight lower than wild-type; however it was not significantly different at the 95% confidence level. The third group contains lines N92046 and N94640 whose mean aerial fresh weight was effectively the same as wild-type. Two phenotypes were identified visually after seven weeks under short-day conditions: 516B12 exhibiting chlorosis and signs of anthocyanin accumulation to a much greater extent than wild-type; 783B02 had accelerated chlorosis of outer rosette leaves, but delayed senescence of inner rosette, with a characteristic banded appearance of senescing leaves.

Considering the senescence analysis, the tilling and T-DNA lines can again be divided into groups according to their response to the temperature treatment. Two senescing phenotypes were identified for the 5°C treatment, the first included all but 783B02 and were characterised by chlorosis proceeding at a slower rate and never reaching the same degree as wild-type. The second included just 783B02 and characterised by accelerated chlorosis at 5°C and reaching a greater than wild-type. There were also three distinct chlorosis onset phenotypes observed in the 5°C treated plants, the first was later onset and included N90558, where chlorosis appeared at most four days later than wild-type. The second included N92046, N94640 and 516B12 where chlorosis onset was identical to wild-type. The third group included 783B02 alone and was characterised by an early start to senescence around 10 days sooner than wild-type. Two principal phenotypic groups were seen in the 20°C treated plants, the first including N90558 and 783B02 characterised by a slower or similar progression of senescence, respectively and both with a lesser degree of senescence compared with wild-type. The second included the rest: N92046, 516B12 and N94640 whose senescence at 20°C proceeded more rapidly or at a similar rate, respectively and both to a greater degree than wild-type. The onset of chlorosis at 20°C was identical in all plant lines to wild-type.

## 5. Discussion

### Aims and approaches

The discovery of the minimal functional *DIN3* promoter narrowed the location of the regulatory sequences, responsible for conferring the dark-responsiveness, to a 50bp portion between 300 and 250bp upstream of the site of transcription initiation (Knight, H., unpublished). It was the aim of this project to identify which cis-acting elements were crucial for the dark-responsiveness by the Fujiki group as well as investigating the contribution of trans-acting factors that have been reported in the literature as being associated with the identified regulatory motifs. The first aim to identify the cis-acting elements was achieved via linker-scan mutation analysis, which replaced 6bp portions of the essential 50bp with an EcoRI site, so that every cis-acting motif would be disrupted by at least one linker-scan line and the effect on expression analysed. The role of candidate trans-acting factors was investigated by observing the effects constitutive over-expression or mutation of the transcription factor gene had on *DIN3* expression and phenotype under limiting growth conditions. In this way, it was the hope of this study to demonstrate the mechanism by which darkness responsiveness is conferred on *DIN3*. Early in the course of the project, qRT-PCR gene expression analysis of *DIN3* revealed that low temperature (5°C) in darkness failed to induce expression. Therefore the aims of the project were extended to include the investigation of which cis- and trans-acting regulatory factors were responsible for the cold repression of *DIN3* expression.

### Regulatory cis-acting motifs and associated transacting factors

#### *MYBS1 / SRMYB (TATCCA/T)*

##### Cis-acting element

The first motif to be disrupted was MYBST1 / MYBS1 (AGGATA/TATCCT) – the putative sugar responsive MYB binding site (SRMYB). The binding site itself was disrupted by scan lines 3 and 4 (Table 3.1.5) and resulted in a loss of dark induction of the *DIN3::LUC* reporter expression (Table 3.6.1). Interestingly, scan line 2 also resulted in a loss of *DIN3::LUC* dark induction without actually disrupting the motif itself. This may be due to disruption of 5' flanking region important for a putative AtMYBS1 homodimer, working in an analogous manner to OsMYBS1 described in rice (Lu et al., 2002). In promoter of rice (*Oryza sativa*)  $\alpha$ -amylase-3 ( *$\alpha$ Amy3*) there is sugar response sequence (SRS) made up of three cis-acting elements: the GC-box, G-box and the

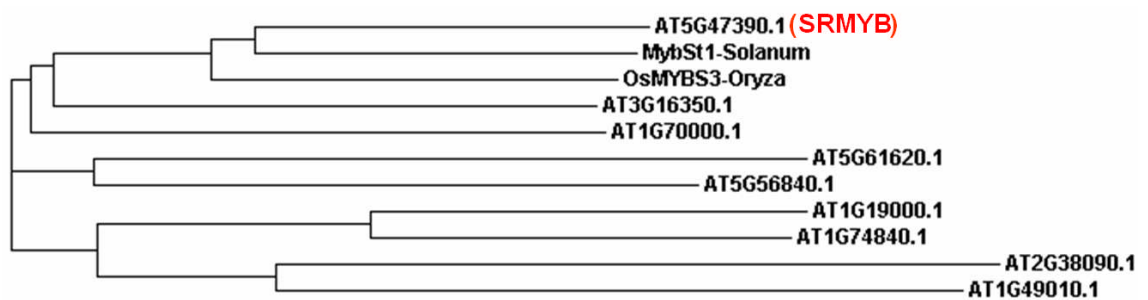
TATCCA element; disruption of any one of these resulted in loss of dark / sugar responsiveness (Lu et al., 1998). Results from scan lines 2, 3 and 4 are consistent with the linker-scan mutation analysis carried out by Lu et al., (1998) on the sugar response sequence (SRS), which contained the TATCCA/T element. A truncated promoter containing just two copies of the GC- and G-boxes still retained responsiveness but much lower than wild-type; the same effect was seen with a promoter containing two TATCCA elements, but with a higher responsiveness. Furthermore as the number of TATCCA elements was increased, there was an almost linear increase in Glc-responsiveness. Linker-scan mutation analysis confirmed the essential nature of the three elements of the SRS (Lu et al., 1998). A version of the *DIN3::LUC* construct with substitution mutations converting the TATCCT element in the *DIN3* -300bp promoter to TAAAAT (fig. 3.7.1), was analysed for altered *DIN3::LUC* expression compared with endogenous *DIN3*. Two out of three of the mutant lines analysed demonstrated wild-type expression patterns (fig. 3.7.2). Additionally, two concatamer lines which had four copies of either TATCCA or TATCCT (*DIN3* type) separated by four bases fused to the 35S CaMV minimal (90bp) promoter (fig. 3.7.1), exhibited no dark-responsiveness (figs. 3.7.3/4). Results using the concatamer lines are consistent with the findings of Lu et al., (1998) that a single element of the SRS is not sufficient to confer dark-responsiveness. However the maintenance of responsiveness following the mutation of the TATCCT element is, *prima facie*, neither consistent with the findings of the linker-scan analysis of this project nor those of Lu et al., (1998) who found that disruption of any one of the SRS elements resulted in loss of induction. However Lu et al., (1998) demonstrated the importance of flanking regions for nuclear factor binding to the TATCCA element in rice, this is supported by results of scan line 2 which result in a loss of dark-induction when it only disrupts the 5' flanking region. It may be that the putative *A. thaliana* SRMYB protein is reliant on the flanking regions and less specific to the TATCCT element itself.

### **Trans-acting factors**

The first trans-acting factor identified as being associated with the MYBS1 / SRMYB motif, was a MYB family transcription factor, MybSt1 (StMYBS1) identified in potato (*Solanum tuberosum*) and with a binding affinity for the motif GGATA (Baranowskij et al., 1994). Reverse complemented this is TATCC, which is obviously the majority of the TATCCA element described by Lu et al., (1998). In the *DIN* genes, the MYBS1 motif in some cases contains a terminal A, T or G. In *DIN3* there is a single MYBS1 site

which reads AGGATA/TATCCT; *DIN6* has four: one TGGATA/TATCCA and three of CCGATA/TATCCG. Three genes encoding three MYBSt1-like proteins have been identified in rice: *OsMYB1*, 2 and 3. The StMYBS1 (Baranowskij et al., 1994) shares 92%, 87% and 85% homology with *OsMYBS3*, 2 and 1 respectively. All three *OsMYBS*s bind to the TATCCA element specifically, with transcript abundance sugar-dependent: *OsMYBS1* and *OsMYBS3* transcripts were in high abundance with low Suc, whereas they exhibited low abundance when Suc was high; the opposite was true for *OsMYB2*. *OsMYBS1* and *OsMYBS2* were revealed to be transcriptional activators of the TATCCA element, whereas *OsMYBS3* acts as a repressor (Lu et al., 2002). More recently it has been demonstrated that *OsMYBS3* functions in the cold signalling pathways and is essential for cold tolerance in rice and acts to induce or repress many genes (Su et al., 2010). However Su et al., (2010) report that *OsMYBS3* responds slowly perhaps too slowly to account for *DIN3* cold-repression should an orthologous protein function in the same manner in *A. thaliana*. Lu et al., (2002) propose that *OsMYBS1*, forming a homodimer, acts as an activator of genes such as  $\alpha$ *Amy3* during sugar starvation, whereas *OsMYBS2* which is much less potent an activator, maintains only a low level of  $\alpha$ *Amy3* when sugar is plentiful, so playing part of the sugar repressed state. *OsMYBS3* transcripts are extremely abundant in senescent levels, where *OsMYBS3* strongly represses  $\alpha$ *Amy3*. In rice it has been established that SnRK1 is essential for expression of *OsMYBS1* and  $\alpha$ *Amy3* under carbon limiting conditions (Lu et al., 2007). An analogous *A. thaliana* MYBS3 protein has been identified, with close homology to MybSt1 and *OsMYBS3* (fig. 5.1) (Knight, M.R. pers. comm.). It has not been established as to whether the SRMYB protein interacts in the same manner as *OsMYB3*. Analysing the effect of over-expression of *SRMYB* on *DIN3* expression, no profound effects were identified: dark induction of *DIN3* was maintained and dark expression levels were only significantly greater than wild-type in one over-expressing line (fig. 4.3.5). Under short-day conditions, *SRMYB* over-expression lines had small, but significant phenotypic effects: a third lower aerial biomass (fig. 4.4.4) and between a quarter and 10% fewer rosette leaves (fig. 4.4.8). Comparing this with short-day grown SRMYB tilling line mutants, those with close to normal levels of *SRMYB* expression (fig. 4.5.1) had significantly lower aerial biomass (516B12 & N90558) (fig. 4.7.1), whereas line 783B02, which had a significantly lower *SRMYB* expression (fig. 4.5.1), had an short-day aerial biomass lower than wild-type but not significantly so (fig. 4.7.1). The over-expression results combined with those of the *SRMYB* mutant lines suggest that levels of *SRMYB* are important in plant productivity or growth rate,

although it is unlikely to be correlated directly to absolute transcript abundance. Over-expression of *SRMYB* resulted in delayed chlorosis compared to wild-type in an 11-day experiment in complete darkness at 20°C (fig. 4.4.13). However there was a variation in starvation phenotype between in the over-expression lines that seemed to correlate, qualitatively at least, with their *SRMYB* expression levels: the over-expressors with the greater *SRMYB* levels exhibited more rapid chlorosis and similar to wild-type. As seen with the other phenotypic experiments, the levels of *SRMYB* transcript do have a simple direct correlation with phenotype. Although *SRMYB* shows homology to the *OsMYBS3* protein (fig. 5.1), results do not implicate *SRMYB* as a repressor of *DIN3*. Fujiki et al., (2000) established that *DIN3* expression was associated with senescence: leaves whose area was 75% yellow had high *DIN3* expression levels. Elevated *DIN3* expression during senescence requires induction, whether this is due to the *SRMYB* transcription factor is unclear, however it seems unlikely that *SRMYB* acts as a repressor in the manner of its rice homologue *OsMYBS3*.



**Figure 5.1 Phylogram of protein sequences with homology to putative *AtMYBS3* (*SRMYB*)**  
Figure reproduced, with permission, from Knight, M.R. (unpublished).

### Contribution to dark and cold-responsiveness

Scan lines 3 and 4 disrupt the motif TATCCT, which shares the core sequence of the MYBS1 binding site in potato (Baranowskij et al., 1994) and rice (Lu et al., 2002), resulting in a loss of dark-responsiveness. In rice the TATCCA motif is an indispensable part of the SRS and together with the other elements can confer dark-responsiveness to previously non-responsive promoters (Lu et al., 1998). The *DIN3* TATCCT element is the putative binding site for an *A. thaliana* homology of MybSt1 and *OsMYB3*. This research indicates that the 5' flanking region is important to the function of this motif, the flanking region is also important in rice TATCCA (Lu et al., 1998). Over-expression of *SRMYB* does not produce a clear dose-dependent phenotype carbon limiting conditions, indicating the role of post-transcriptional regulation in *SRMYB* and a more complex relationship between *SRMYB* levels and *DIN3* regulation. No evidence was found to support a role for *SRMYB* acting as a repressor in the manner of *OsMYB3* its

closest rice homologue. Together these results indicate that the TATCCT element, as part of a putative SRS in *DIN3*, does not regulate dark-responsiveness alone, which is consistent with conclusions of Lu et al., (1998) on SRS function. The linker-scan mutation analysis in this project did not identify a single instance of a loss of cold repression. The introduced linker-scan mutations only ever resulted in a complete loss of induction (Table 3.6.1). The literature ascribes no role for MYBS1/2/3 transcription factors in the regulation of cold-responsive transcription. Low temperature has been reported to induce an accumulation of hexose and hexose phosphates (Kaplan et al., 2007), causing the inactivation of SnRK1 (Zhang et al., 2009), which has a role in the Glc-repression of *MYBS1* expression and the activation of MYBS1 (Lu et al., 2007).

### **ABRERATCAL and CGCG-box**

#### **Cis-acting element**

Scan line 5 disrupted the next downstream motifs, which shared a core CGCG motif: ABRERATCAL and the CGCG-box (Table 3.1.5). No *DIN3::LUC* expression could be detected from the seedlings of scan line 5 (Table 3.6.1; fig. 3.5.6), which indicates that the linker-scan mutation disrupts a crucial cis-acting element. Scan line 6 also disrupts the ABRERATCAL and CGCG-box; there is no dark induction of *DIN3::LUC* expression in scan line 6. These elements have dark and cold regulatory functions; the dark-responsiveness will be considered first, followed by low temperature regulation. Disruption of either the ABRERATCAL or CGCG-box, two motifs associated with ABA-dependent (Doherty et al., 2009) and –independent (Yang & Poovaiah, 2002) cold response pathways, resulted in a loss of dark-induction of *DIN3::LUC*. The other component element of the SRS is the G-box (ACGT) (Lu et al., 1998), whilst this specific element is not found in the promoter of *DIN3*, however a similar and related element the ABRE ‘coupling element 3’ (CE3), which has the consensus sequence (ACGCGTG) (Choi et al., 2000), is (Table 3.1.4). The CE3 type ABRE is over-represented in rice, but is very rare in *A.thaliana* (Gómez-Porrás et al., 2007); the same study found the CE3 motif in the promoter of *DIN3*, confirming the finding of this project. CE3 were found to be associated with G-box ABREs (Suzuki et al., 2005) and an earlier study found that CE3 and G-box ABRE are functionally equivalent (Hobo et al., 1999). The literature indicates that S1-group bZIP transcription factors have a role in activating genes such as *ProDH* during sugar starvation (Hanson et al., 2008), however their specificity is to a G-box (ACGT) (Kang et al., 2010), not the CE3 motif (ACGCGTG) (Choi et al., 2000; Doherty et al., 2009) found in *DIN3* at the loci of dark

non-responsive scan lines 5 and 6. It may be there is an, as yet undiscovered, kinase-dependent bZIP transcription factor with specificity to the CE3 motif. If so, this would give *DIN3* a variant of the classical SRS with a TATCCT, rather than a TATCCA and a CE3 motif rather than a G-box. Apart from two base changes, this is exactly what is found in *DIN3* and the linker-scan mutation analysis carried in this project produced similar results to that of Lu et al., (1998) – that disruption of one or other members of the SRS results in loss of dark/sugar responsiveness. The ABRERATCAL / ABRE – Abscic acid responsive element, has been implicated in ABA signalling and annotated as a cold-regulatory motif. Evidence for a cis-acting element mediating ABA responsive gene expression was discovered in wheat with the core consensus motif: ACGT (Marcotte Jr et al., 1989). More recent work in *A. thaliana* has revealed a greater degree of degeneracy in the ABRE, which has the consensus (C/A)ACG(T/C)G(T/G/C) (Kaplan et al., 2006) and so includes the version found in *DIN3*. Doherty et al., (2009) found evidence that the ABRE-CE3 motif had cold-repressive properties. The CGCGBOXAT / CG-1 Box motif, which was identified as member of a common set of regulatory motif found in the promoters of cold response transcription factors CBF2 and ZAT12 (Doherty et al., 2009). CBF2 and ZAT12 bind the C-repeat/Dehydration responsive element (CRT/DRE) found in the promoters of, amongst others (Vogel et al., 2004), COR genes which mediate cold acclimation and freezing tolerance (Thomashow, 1998). One of the six conserved motifs (CM2) identified by Doherty et al., (2009) contains the core motif of the AtSR1 (CAMTA3) binding site, a calcium-dependent calmodulin (CaM) binding protein. The optimal motif sequence for AtSR1 is ACGCGG, but it also binds ACGCGT (Yang & Poovaiah, 2002), which is the sequence of the CGCG motif found in the promoter of *DIN3* (Table 3.1.4); this is found within the ABRERATCAL motif. The core CGCG motif was detected in all other *DIN* genes, except *DIN1* (Table 3.1.1). As commented above, the linker-scan analysis did not identify a single instance of loss of cold-repression independent of a loss of dark-induction. Despite the literature ascribing cold regulation to the ABRE-CE3 and CGCG-box motifs, results from this project could not implicate the cis-acting elements conclusively in cold regulation.

### **Trans-acting factors**

The ABRERATCAL and CGCG-box motifs typically constitute a G-box, which is bound by bZIP transcription factors. bZIP11 and 53, which are members of the S1 group of bZIP transcription factors have been implicated in abiotic stress responses particularly hypoosmolarity following recovery from drought, salinity or freezing

stresses (Jakoby et al., 2002; Satoh et al., 2004; Weltmeier et al., 2006). More recently it has been established that *bZIP1* (AT5G49450), another S1 group member, is repressed by Glc in a fast, sensitive and reversible manner (Kang et al., 2010). Kang et al., (2010) demonstrate that bZIP1 binds to the ACGT core motif and other bZIP family transcription factors also recognise the ACGT core motif (Thomas, 1993). As hypothesised above, it may be that an uncharacterised bZIP transcription factor exists with specificity for the ABRE-CE3 found in *DIN3*. Transgenic plants were created over-expressing *bZIP1*, to investigate the role of this transcription factor in the ABRE and CGCG-box motifs indicated as important in dark-responsiveness by the linker-scan mutation analysis. Of three *bZIP1* over-expressing lines, all were found to exhibit dark-induced *DIN3* expression at significantly greater levels, between one-and-a-half and two times greater than wild-type (fig. 4.3.1). The over-expression of *bZIP1* also resulted in a significantly lower, around a half, aerial biomass (fig. 4.4.1) and a fifth fewer rosette leaves (fig. 4.4.5) than wild-type. Qualitatively, the *bZIP1* over-expressor phenotype was characterised by an elongated petiole with circular, spoon-shaped leaves (fig. 4.4.9: A & B). Two out of three *bZIP1* over-expressing lines had delayed chlorosis after 11 days in complete darkness (fig. 4.4.10). Superficially, the elongated petioles and change in leaf morphology resembles the shade avoidance phenotype (Smith & Whitelam, 1997). The stunted growth and improved tolerance to prolonged darkness in the *bZIP1* over-expressors, appear to provide further evidence that S1 group bZIPs are involved in metabolite status signal transduction, thereby influencing plant growth and development (Kang et al., 2010; Hanson et al., 2008). Two genes up-regulated during carbon limiting conditions, coding for *Asparagine synthase1* (*ASN1*) and Pro dehydrogenase (*ProDH*) are rapidly induced by bZIP11. More generally, the majority of genes induced by bZIP11 are repressed by sugar and the majority of bZIP11-repressed genes are induced by sugar (Hanson et al., 2008). As the expression and activation of *OsMYBS1/2/3* are regulated by SnRK1 (Lu et al., 2007), the S1 group bZIPs work synergistically with KIN10/11 (SnRK1) to regulator *DIN6* (Baena-González et al., 2007). The role of the sugar sensing kinase, HXK in *DIN3* regulation is not an essential one; no impairment of dark-induction of *DIN3* expression was identified using the HXK null mutant *gin2-1* (fig. 3.8.1), as with *DIN6* (Baena-González et al., 2007).

Given the presence of CGCG-box motifs in the promoters of *CBF* genes and the role of ABA signalling in *CBF* expression, *CBF1* over-expressing lines were generated. The one confirmed transformant line had a lower dark-induced *DIN3::LUC* expression than wild-type however this difference was not significant (fig. 4.3.2). The over-expression of *CBF1* resulted in a large and significant reduction, by an 80%, in aerial biomass (fig. 4.4.2) and third fewer rosette leaves (fig. 4.4.6). Qualitatively, *CBF1* over-expressors appeared stunted in their growth habit, with low vertical height, small rosettes and dark green leaves compared with wild-type; otherwise leaf morphology appeared normal (fig. 4.4.9: D). These observations accord with those made in the literature (Gilmour et al., 2004). After 11 days in complete darkness, very little chlorosis was seen in plants over-expression *CBF1* (fig. 4.4.11), the effect this over-expression is analogous to wild-type plants kept in darkness at 5°C (fig. 4.7.3). CaM and Ca<sup>2+</sup> signalling have been implicated in early low temperature perception (Knight et al., 1991; Kaplan et al., 2006). A family of Ca<sup>2+</sup>-dependent CaM binding proteins, the CaM transcriptional activators (CAMTA) were found to preferentially bind the CGCG motif (Bouché et al., 2002). Doherty et al., (2009) confirmed CAMTA binding affinity for the CGCG motif and further demonstrated that CAMTA could induce expression of *CBF2*; Doherty et al., (2009) reported the impaired freezing tolerance of *camta1camta3* double mutants. The CGCG motif and other conserved motifs identified by Doherty et al., (2009) were over-represented in the promoters of early cold-responsive genes and the CGCG motif, along with three others, exhibit transcriptional repression properties. An attempt was made to generate *CAMTA1* over-expressing plants, however no transformants were identified. This follows the earlier failed attempts at producing *CAMTA1* over-expression lines, in which it was postulated that *CAMTA1* over-expression is lethal (Boyce, Knight & Fromm, unpublished data). Mutants of *camta3* (*AtSR1*) have higher biotic stress tolerance than wild-type, thus implicating CAMTA3 as a repressor of transcription (Galon et al., 2008). CAMTA1 was show to be a transcriptional regulator of abiotic stress-responsive genes cause the up- and down-regulation of many genes (Galon et al., 2010).

### **Contribution to dark and cold-responsiveness**

Scan lines 5 and 6 disrupt the ABRERATCAL and the CGCG-box elements which share a common CGCG core motif (Table 3.1.5). In both these scan lines no dark-induction of *DIN3::LUC* expression was seen (Table 3.6.1). The SRS described by Lu et al., (1998) includes a G-box motif, whilst this is not found in the *DIN3* promoter, the ABRE-CE3 motif is, which is functionally equivalent to a G-box (Hobo et al., 1999;

Choi et al., 2000). As the TATCCT motif seems to be a variant of the TATCCA in rice, so the ABRE-CE3 could be a variant of the G-box element. These variants do not depart greatly from the Lu et al., (1998) model of the SRS and so the TATCCT and ABRE-CE3 elements could constitute part of a SRS in *DIN3*. The loss of dark-induction following the disruption of either of these two elements accords with the original findings of Lu et al., (1998) that all the elements of the SRS are necessary. Using an *ABRE::LUC* construct used in Kaplan et al., (2006), no difference was seen between light or dark treated gene expression (data not shown). This confirms Lu et al., (1998) finding that no single element can confer dark-responsiveness. The literature also ascribes cold-responsiveness to these elements; ABA induces *CBF* expression, factors which regulate *COR* genes (Knight et al., 2004) and the CGCG motif is over-represented in cold-regulated genes (Doherty et al., 2009). The ABRE-CE3 found in *DIN3* is bound by an ABRE-binding protein in rice (RoyChoudhury et al., 2008), where the motif is common (Gómez-Porrás et al., 2007). The linker-scan mutation analysis was unable to establish a role for these elements in the cold-responsive of *DIN3*. However the ABRE-CE3 motif, found in *DIN3*, has been found to have transcription repression properties (Doherty et al., 2009). The S1 group bZIP transcription factors are implicated in abiotic stress (Jakoby et al., 2002; Satoh et al., 2004; Weltmeier et al., 2006), they are also sugar repressed (Kang et al., 2010; Hanson et al., 2008). Over-expression of *bZIP1* up-regulates *DIN3* expression and significantly alters the growth phenotype and delays the development of chlorosis during complete darkness. Whilst this project has not shown that bZIP1 binds to the ABRE-CE3 motif and to-date S1 group bZIPs are only known to bind to G-boxes (ACGT core), it has established that it is required for dark-induction of *DIN3*. It may be that there is an as yet undiscovered factor which binds to the ABRE-CE3 in *DIN3*. As for bZIP1 it may exact its effect up- or downstream of the crucial 50bp. Regrettably no *CAMTA1* over-expressing lines were generated, however *CAMTA1* has been shown to be a transcriptional repressor during abiotic stress (Galon et al., 2010) with affinity for ABRE-CE3 (Kaplan et al., 2006), which given that all other trans-acting factors with affinity for ABRE-CE3 are transcriptional activators (Pandey et al., 2005) makes *CAMTA1* a good candidate for cold-repression meriting further investigation. Over-expression of *CBF*, a regulator of the *COR* and other cold-responsive, did result in a reduction in *DIN3* expression, but not significantly so. The CRT/DRE motif, to which CBFs bind, was not present in the crucial 50bp of the *DIN3* promoter, but it was found further downstream of this region (Table 3.1.1) and therefore undisrupted by the linker-scan mutation analysis.

## **MYB2CONSENSUSAT**

### **Cis-acting element**

Scan lines 7 and 8 disrupt the MYB2CONSENSUSAT element, the form found in *DIN3* (5' → 3': CAACGG) (Table 3.1.5) which is bound by the AtMYB2 transcription factor (Abe et al., 2003). Only dark-induction of *DIN3::LUC* was identified in scan line 7, but not in scan line 8; no dark-induction of *DIN3::LUC* was exhibited by scan line 9 seedlings (Table 3.6.1). The element is found on the opposite strand, which together with the linker-scan mutation analysis, suggesting that the 5' portion and its flanking region is more important for *DIN3* dark-induction. The AtMYB2 transcription factor binding site is required for ABA-mediated drought-induction of *rd22* (Abe et al., 2003). The other ABA associated cis-acting element in *DIN3*, ABRE-CE3, also results in loss of dark-induction when disrupted by the linker-scan mutation analysis. The consensus sequence for the AtMYB2 binding motif is fairly degenerate (A/T AACCA or C/T AAC G/T G), which like the ABRE-CE3 / CGCG-box may permit the binding of an unknown transcription factor. It may be the third element of a putative *DIN3* SRS: Lu et al., (1998) originally described three essential motifs constituting a SRS. Together scan lines 8 and 9 disrupt a common sequence: AGTGTG (5' → 3': CACACT), which on the opposite strand could be read as a MYC binding site (CANNTG) (Shinwari et al., 1998). This putative motif bears a vague resemblance to a gibberellic acid response element (GARE), which in *A. thaliana* reads CAACTGTC and is bound by GA-responsive MYB-family transcriptional activators. GAREs are found in the promoters of  $\alpha$ -amylase genes (Gocal et al., 2001). In rice, genes with GAREs are GA-induced and ABA-repressed (Sutoh & Yamauchi, 2003), which is analogous to the expression patterns of *DIN3*. A GARE motif is also shown in the promoter of  *$\alpha$ Amy7* in the analysis by Lu et al., (1998). The approach taken by this project did not reveal a role for this element in cold-repression of *DIN3*. However cold-regulatory roles may emerge from consideration of associated transcription factors below.

### **Trans-acting factors**

The only known transcription factor that binds the identified cis-acting element disrupted by the linker-scan mutation analysis is AtMYB2. It is a Ca<sup>2+</sup>-dependent CaM binding protein, whose affinity for the cis-acting element is enhanced by this interaction (Yoo et al., 2005). Yoo et al., (2005) report that AtMYB2 induces the expression of salt- and dehydration-responsive genes such as *rd22* and  $\Delta^1$ -pyrroline-5-carboxylate synthetase (*P5CS*), the latter responsible for Pro biosynthesis; Pro is a compatible solute

that accumulates during dehydration stress caused by salt, drought or freezing stress (Anchordoguy et al., 1987). Despite its potential link to cold-responsive genes, the literature reports nothing to suggest that AtMYB2 acts as a transcriptional-repressor. A similar conclusion can be made about the putative MYC binding site disrupted by scan lines 8 and 9; the AtMYC2 transcription factor is a transcriptional activator, responsive to drought and salinity (Abe et al., 2003). No direct links to low temperature or transcriptional repression have been reported. The GARE is found in the promoter of *αAmy7* (Lu et al., 1998), however the MYB transcription factors that bind to the GARE are implicated in GA-dependent germination and flowering (Gocal et al., 2001).

### **Contribution to dark and cold-responsiveness**

The region disrupted by scan lines 7, 8 and 9 certainly contribute towards dark-responsiveness (Tables 3.6.1). As with the rest of the linker-scan mutation analysis results, the contribution of this region to cold-responsiveness could not be identified. The region disrupted by scan lines 7 and 8 the MYB2CONSENSUSAT element, found on the opposite strand, which is the binding site for AtMYB2 (Abe et al., 2003). The linker-scan mutation analysis indicated that the 5' portion of this element was the most important, since scan line 7 retained dark-induction of *DIN3::LUC*. The common sequence disrupted by scan lines 8 and 9, on the opposite strand, is the binding site for AtMYC2 (Shinwari et al., 1998). Both AtMYB2 and AtMYC2 are transcriptional activators, responsive to drought and salinity stresses, with no direct links to cold regulation (Abe et al., 2003). The immediate relevance to *DIN3* is unclear. The common scan line 8 and 9 sequence bears vague resemblance to a GARE, but the associated MYB transcription factors are only implicated in GA-dependent, regulation of germination and flowering (Gocal et al., 2001). This site may be the target of a yet uncharacterised transcription factor within the MYB or bHLH family, which mediates the dark-responsive properties of this region. What is certain is that this is the third portion of the crucial 50bp of the *DIN3* that has been confirmed to be required for dark-induction and it likely the third element in a putative *DIN3* SRS fitting the model postulated by Lu et al., (1998).

### **Other cis-acting elements**

Scan line 4 also disrupts a GATA, which is part of the TATCCT motif, however despite the prevalence of LREs in the -500bp promoter region of the *DIN* genes, the significance of their contribution to *DIN* gene light/dark regulation is not likely to be great given the very conclusive experiments of Fujiki et al., (2000) with photosynthesis

inhibitors producing *DIN* gene induction in the light. This is further supported by the disruption of another GATA motif not part of a SRMYB motif by scan lines 11 and 12 (Table 3.1.5), which results in no loss of dark induction of *DIN3::LUC* (Table 3.6.1). Therefore GATA is unlikely to be functioning as a light responsive element in *DIN3*. Over-expression of *GATA21* transcription appeared to cause a loss of dark-induction, however in none of the transformant lines was this significantly different from wild-type (fig. 4.3.3). The *GATA21* over-expressor exhibited a significantly lower, by around a third, aerial biomass than wild-type (fig. 4.4.3); in one line there was a significant, albeit small, reduction in the number rosette leaves (fig. 4.4.7). No visual short-day grown phenotype was identified in *GATA21* over-expressors (fig. 4.4.9: E – G), neither was there a significant effect on development of chlorosis during complete darkness treatment (fig. 4.4.12). The role of the *GATA21* transcription factor in dark-regulation is inconclusive from the over-expression study.

### **Conclusions**

This project demonstrated that the -300bp minimal promoter contains all the essential cis-acting elements require for dark-induction and cold-repression of *DIN3* expression. The linker-scan mutation analysis combined with the *in silico* analysis revealed that three cis-acting elements: TATCCT, ABRE-CE3 and putative AtMYB2/AtMYC2 binding site are indispensable for the dark-induction of *DIN3*. These constitute a putative *DIN3* SRS. The approach taken by this project did not identify any cis-acting elements that when disrupted, manifested a loss a cold-repression in the expression of *DIN3::LUC* reporter. Analysis of candidate trans-acting factors for a potential role in mediating the dark- and cold-responsiveness of *DIN3* indicated that the putative *A. thaliana* orthologue of the TATCCA-specific *O. Sativa* MYBS3, SRMYB, had a role in dark-responsiveness but this effect was not a direct dose-dependent one, indicating the importance of other cis- and trans-acting factors. Over-expression of *bZIP1* increased the overall expression of *DIN3* and significantly modified the growth habit and dark-induced starvation response. However bZIP1 has not been described as having affinity for ABRE-CE3 motif, opening the possibility of an unknown transcription factor. No evidence was found of direct role for the other transcription factors in the regulation of *DIN3*.

There are two possible conclusions from the available results; the first is that the dark/sugar responsive and cold responsive regulatory motifs are shared. This is certainly

borne by the annotation of the cis-acting elements in the literature. Focussing on the ABRE-CE3/CGCG-box the best candidate for cold regulation in the *DIN3* promoter, this motif is also shown to be required for dark-induction and is a putative non-G-box motif as part of a putative SRS in *DIN3*. This particular motif would likely be bound by some S1-group bZIP transcription factor. bZIP11, a member of the S1-group, is repressed by dehydration stress (Kiyosue et al., 1996), so there would be no competition for this motif should an ABRE-binding protein such as CAMTA wish to bind. At the same time low temperature has been reported to induce an accumulation of hexose and hexose phosphates (Kaplan et al., 2007), causing the inactivation of SnRK1 (Zhang et al., 2009), which has a role in the Glc-repression of S1-group bZIPs (Baena-González et al., 2007) and the activation of OsMYBS1 and OsMYBS1 expression (Lu et al., 2007); HXK may play a limited role in the Glc-repression of *DIN3* (Baena-González et al., 2007). The elevated hexose levels during low temperature mean that the motifs of a putative *DIN3* SRS will be unoccupied for transcriptional repressors to bind at the same site. The second interpretation, which is more parsimonious, would be that there is no binding of a cold-induced transcriptional repressor, but instead cold-repression is merely lack of induction due to sugar-repression of the trans-acting factors: the S1-group bZIPs and MYBS1. Indeed, for there is no evidence of the binding of any trans-acting factor to bring about sugar-repression of *DIN3*. This is attested to by the gene expression analysis (qRT-PCR) that demonstrates no significant difference between light *DIN3::LUC* expression and cold-inhibition and *DIN3* cold expression is not significantly lower than that during the light (fig. 3.6.1). Fujiki et al., (2001) found no difference between light-repressed and dark sugar-repressed *DIN3* expression.

### ***Further work and improvements***

The most seriously short coming of this project was its inability to identify conclusively a cis-acting element or trans-acting factor responsible for the cold-repression identified. It may be suggested above, that there is a cold-responsive trans-acting factor that shares one of the dark-responsive cis-acting elements in which case the linker-scan mutation analysis would not be able to decouple the two responses. Alternatively, there may be an element downstream of the 50bp that mediates the cold-repression. Indeed there is a CRT/DRE motif downstream of this portion of the promoter. As Su et al., (2010) report in rice, a cold repressive OsMYBS3 orthologous may be too slow to account for the rapid cold-repression of *DIN3*; Su et al., (2010) place the DREB1/CBF pathway in the rapid response cold signalling model. The role of a downstream CRT/DRE motif in cold-repression of *DIN3* merits further investigation. The *in silico* analysis indicated the

presence of a CATMA binding site – ABRE-CE3, but the generation of a viable CAMTA over-expressing lines has provided in effective. Further work to assess the contribution of CATMA to *DIN3* cold-regulation could make use of an inducible promoter so that potential lethality associated with constitutive expression can be avoided. The use monitoring of the effects of dark-induced starvation was crude and qualitative at best. If this particular experiment were to be repeat use of a fluorimeter to record the photosynthetic capacity of photosystem II and providing a Fv/Fm measurement (Oxborough, 2004; Maxwell & Johnson, 2000) may yield more conclusive results.

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