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Identification of genome targets of the DET1 complex in higher plants

Thesis

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IDENTIFICATION OF GENOME TARGETS OF THE DET1 COMPLEX IN HIGHER PLANTS

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Abstract

Light provides a major source of information from the environment during plant growth and development. Light-regulated gene expression is partly controlled by the phytochrome photoreceptors, which once activated, are imported into the nucleus where they bind and activate transcription factors such as PIF3. Coupled with this, the degradation of positively acting intermediates such as the transcription factor HY5 by COP1 and other ubiquitin ligases acts to repress photomorphogenesis in darkness. Another negative regulator of photomorphogenesis is DET1 (De-Etiolated 1), which forms part of a nuclear-localised complex with the plant homolog of UV-Damaged DNA Binding protein 1 (DDB1) and the E2 ubiquitin-ligase activating protein COP10 (Constitutive Photomorphogenic 10). Previously it was found that DET1 binds chromatin via a direct interaction with the core histone H2B, suggesting that DET1 may repress light activated genes by interacting directly with their promoters.

Here it is shown that DET1 forms part of a CUL4-based ubiquitin ligase complex and is localised to discrete foci throughout the nucleus. Chromatin Immunoprecipitation experiments show that in the dark DET1 binds to the promoters of the light induced *CAB2* and *HEMA1* genes, and that this binding is abolished in the light, coincidental with the activation of these genes. DET1 was also detected at the promoter of the light-repressed *POR-A* gene when it is in its repressed state as well as the promoter of the developmentally regulated *FT* gene, again when this gene is repressed. Based on these data it is proposed that the DET1 complex binds the promoters of light-regulated genes in the dark and directly represses their transcription, either by chromatin remodelling and/or ubiquitin-mediated regulation of the transcriptional apparatus. Light causes the dissociation of the DET1 complex from these promoters, allowing activation of these genes. DET1 may also play a more general role in genome maintenance during plant development.

Contributions

All the experiments described in this thesis are my own work except for the Yeast-2-Hybrid and GST-pulldown experiments described in section 3.2.3 and Fig 3.6a-c which were preformed by Anne Bernhardt (Free University of Berlin, Germany)

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Annotations

Modifications of core histones are annotated as described by (Turner, 2005). Briefly the order is: Histone type - modified residue - type of modification - level of modification. For example H3K9me2 denotes the core histone H3 di-methylated at lysine 9.

Protein nomenclature for phytochrome and phototropin photoreceptors is described in (Quail et al., 1994) and (Briggs et al., 2001) respectively. Specifically, the apoprotein is denoted in capital letters (PHY/PHOT) while the active photoreceptor consisting of the chromophore conjugated to the apoprotein is denoted in lower case letters (phy/phot). This convention has not been widely adopted for the cryptochrome photoreceptor and thus the conventional nomenclature is used here (uppercase; wild type, lower case; mutant).

Abbreviations

AEBSF:	4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride
BSA:	bovine serum albumin
$CaCl_2$:	calcium chloride
CCD:	charge-coupled device
CFP:	cyan fluorescent protein
DAPI:	4',6-diamidino-2-phenylindole
DDW:	double distilled water
DNA:	deoxyribonucleic acid
ECL:	enhanced chemiluminescent reagent
EDTA:	ethylenediaminetetraacetic acid
EGTA:	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
GFP:	green fluorescent protein
GST:	glutathione S-transferase
FRET:	flourecence resonance energy transfer
HA:	Influenza hemagglutinin epitope tag
HEPES:	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HCl:	hydrochloric acid
kb:	kilo base
kDa:	kilo Dalton
MES:	2-(N-morpholino)ethanesulfonic acid
NaCl:	sodium chloride
NP40:	Igepal CA-630 detergent
$OD_{600\,nm}$:	optical density at 600 nm
PBS:	phosphate-buffered saline
PCR:	polymerase chain reaction
PIPES:	piperazine-N,N'-bis(ethanesulfonic acid)
PVDF:	polyvinylidene fluoride
SDS:	sodium dodecylsulfate
SDS-PAGE:	sodium dodecylsulphate polyacrylamide gel electrophoresis
Tris-HCL:	tris (hydroxymethyl) aminomethane hydrochloride
YFP:	yellow fluorescent protein

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1. Introduction

1.1 Photomorphogenesis

Unlike animals that can move to a more favourable location or alter their behaviour in order to adapt to their environment, plants are sessile organisms which cannot move and so have to adapt to their location and prevailing environmental conditions.

To do this they have evolved mechanisms to accurately sense the environment around them and alter their developmental programmes in order to adopt the optimal "body-plan" for the prevailing conditions (Casal et al., 2004; Meyerowitz, 2002). Most plants use light as their primary energy source, so it is not surprising that they are acutely sensitive to this environmental signal, being able to detect even faint starlight, direction of light and the relative spectral composition of the light they receive in a spectrum stretching from the ultra-violet to infra-red (Mustilli and Bowler, 1997) (Quail, 2002a) (Gyula et al., 2003).

When a seed germinates in the absence of light, for example if it is under soil or leaf litter, it adopts a developmental program known as skotomorphogenesis. The hypocotyl grows upward using the gravity field to orient itself and rapidly extends (by cell expansion) in order to reach the surface and light before the internal energy stores of the seedling are exhausted. In order to avoid damage, the cotyledons remain closed and folded back against the hypocotyl resulting in a pronounced apical hook. The etioplasts (undifferentiated chloroplasts) remain undeveloped and the seedlings are white or yellow in colour. This growth pattern is also known as etiolated growth (Schafer and Nagy, 2006).

When the seedling reaches the surface and its photoreceptors detect the presence of light they trigger photomorphogenesis (light controlled growth/development) and the seedling undergoes a developmental transition known as de-etiolation. In this process the cotyledons open, hypocotyl elongation is inhibited, photosynthesis begins and cell differentiation is initiated in the vegetative meristems in order to produce true leaves. These changes appear to be mediated largely by light-induced changes in gene expression which result in differential expression of almost 1/3 of the genome (about 8000 genes; Ma et al., 2001; Tepperman et al., 2001; Schroeder et al., 2002).

1.2 Light signalling mutants

Plant responses to light quality and direction have been studied since the 1700's and the existence of a light sensing pigment known as phytochrome has been known since the 1960's (Schafer and Nagy, 2006). However photomorphogenesis research really took off in the early 1980's with the application of molecular genetic methods to the model plant *Arabidopsis thaliana*. In these early experiments mutagenised seedlings were grown under strong light and several mutants with impaired photomorphogenic responses, e.g., insensitivity to the repression of hypocotyl elongation by light were isolated (Koornneef et al., 1980). These *hy* (hypocotyl elongated) mutant loci were later cloned and found to encode several photoreceptors (*HY3* and *HY4*), enzymes responsible for the biosynthesis of the photoreceptor chromophore (*HY1* and *HY2*) and a transcription factor that acts downstream of the photoreceptors (*HY5*) (Schafer and Nagy, 2006).

Other screens were performed by looking for mutants that spontaneously initiated photomorphogenic development in the absence of light, the *cop* (constitutive photomorphogenesis) and *det* (de-etiolated) mutants (Deng et al., 1991; Chory et al., 1989). Cloning of these loci identified a number of negative regulators of light signalling. Additional screens for mutants with impaired phototropic responses (orientation of growth towards light) identified the *nph* (non phototropic hypocotyl) mutants (Liscum and Briggs, 1995), which were later shown to encode several additional photoreceptors and light signalling components. More recently many more components of light signalling pathways have been identified using yeast-2-hybird screens or based on homology (Ni et al., 1998; Fankhauser et al., 1999; Khanna et al., 2004). The study of these proteins and their mutants has allowed great progress to be made in understanding the molecular mechanisms and pathways controlling photomorphogenesis.

1.3 Photoreceptors

Plants sense light using a small group of light-absorbing/sensing proteins known as photoreceptors. These consist of the red/far-red light-sensing phytochromes, the UV-A/blue light-sensing cryptochromes and phototropin photoreceptors (Lin, 2002; Quail, 2002b). Recently a small family of putative blue-light sensing photoreceptors typified by FKF1 (Flavin-binding Kelch repeat F-box 1) have been identified (Somers et al.,

2000). Plants also sense UV-B light, but no photoreceptor sensitive to this wavelength has yet been identified (Ulm and Nagy, 2005).

1.3.1 Phytochromes

Phytochromes are encoded by small muiltgene families, e.g., *Arabidopsis* has five members, *PHYA-E* (Clack et al., 1994; Sharrock and Quail, 1989). The phy proteins they encode regulate many processes such as seed germination, de-etiolation, plant development and architecture, shade avoidance responses, regulation of the circadian clock and control of the vegetative to floral transition (Franklin and Whitelam, 2006). The approximately 125 kDa PHY apoprotein is conjugated to a linear tetrapyrrole chromophore, phytochromobilin, at a conserved cysteine in the N-terminal bilin-lyase domain (Furuya and Song, 1994). The C-terminus contains a PAS-related dimerisation-domain and a histidine-kinase related catalytic domain (HKRD) (Furuya and Song, 1994).

Phytochromes exist as homodimers (although some evidence of heterodimerization exists; (Sharrock and Clack, 2004) and can exist in two photointerconvertable forms, Pr (red light-absorbing) and Pfr (far-red light-absorbing). In the absence of light phytochromes are in their inactive Pr conformation. Absorption of red light causes phytochromes to convert to the active Pfr form.

Earlier biophysical characterisation identified two pools of phytochrome, a light labile pool which was rapidly degraded in the presence of light, known as type I phytochrome and subsequently shown to be phyA, and a light stable pool which is stable in the presence of light and known as type II, shown to consist of phyB-E (Quail et al., 1995). phyA is the most abundant phytochrome in dark grown seedlings, making up about 85% of the total phytochrome pool (Sharrock and Clack, 2002), but it is rapidly degraded in the presence of light by the COP1 ubiquitin-ligase (Seo et al., 2004). This results in phyB being the most abundant phytochrome in light-grown plants, with phyC-E present in smaller amounts (Sharrock and Clack, 2002).

Phytochrome responses to light can be classified into 3 types in accordance to the different light intensities at which they occur, the very low fluence responses (VLFR), the low fluence responses (LFR) and the high irradiance responses (HIR). VLFR is mediated exclusively by phyA, while the LFR is mediated largely by phyB with phyC-E also playing minor roles. Red light HIR is mediated by phyB while the far-red HIR is mediated exclusively by phyA which is thought to convert from the Pfr form to another active form termed Pr+ that transduces the high irradiance response

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(Shinomura et al., 2000). In general phyA is the major sensor of far-red light while phyB (together with phyC-E) senses red light.

1.3.2 Phytochrome localisation and signalling

Early results using cellular fractionation and immunological techniques suggested that phytochromes were cytosolic proteins and were possibly membrane bound (Bowler and Chua, 1994). While this is true in the dark it has more recently been shown that following activation by light the phytochromes translocate to the nucleus (Kircher et al., 2002; Kircher et al., 1999; Sakamoto and Nagatani, 1996). This import is light-quality/quantity dependent and the rate of import depends on the ratio of Pr to Pfr. Furthermore, phyA is imported rapidly (maximal after -10min) while the type II phytochromes (PhyB-E) are imported more slowly (1-8 hours) (Kircher et al., 2002).

It appears that changes in protein conformation that result from conversion from the Pr to the Pfr form unmasks a nuclear import signal in the hinge region between the N and C-terminal domains (Chen et al., 2005) resulting in nuclear import. Once in the nucleus phytochromes form discrete nuclear speckles that appear important for function. In the case of phyB, it first localises to transient "early" speckles that disappear over 10-20 min and then after about 30 min it relocalizes to larger, more stable "late" speckles (Bauer et al., 2004). Speckle formation appears to be a fluence dependent process and it has been proposed that the Pr:Pfr heterodimer is sufficient for nuclear import while the Pfr:Pfr homodimer forms nuclear speckles (Chen et al., 2003). Biochemical purification suggests that these "late" speckles are the plant equivalent of animal interchromatin granule clusters (ICGs) (Schafer et al., 2006). In animals, ICG's appear to be involved in processes related to RNA splicing and transcription, which in turn suggests that phytochromes may directly regulate not only transcription but also RNA editing. While these results suggest that phytochrome signal transduction occurs mainly in the nucleus, a significant portion of phytochrome remains in the cytoplasm under all light conditions (Kircher et al., 2002) and a number of proteins that interact with and/or that may transduce phytochrome signals are cytoplasmic (Fankhauser et al., 1999; Guo et al., 2001).

1.3.3 Phytochrome signalling in the cytoplasm

Early microinjection studies suggested that phytochromes may act via signalling cascades involving heterotrimeric G-proteins, calcium and calmodulin (Bowler et al., 1994a) (Bowler et al., 1994b). However sequencing of the Arabidopsis genome showed that both $G\alpha$ and $G\beta$ were present as single copy genes and that overexpression or null mutants of these gene had no effect on light signalling (Jones

et al., 2003). Support for calcium involvement in light signalling came from the cloning of *SUB1* (short under blue1), a calcium binding EF hand protein (Guo et al., 2001). SUB1 localises to the nuclear envelope and/or endoplasmic reticulum and appears to act as a negative regulator of both phyA and cryptochrome signalling (Guo et al., 2001).

The only constitutively cytoplasmically localised phytochrome interacting protein identified to date is PKS1 (Phytochrome kinase substrate 1), which binds phyA and phyB and is phosphorylated *in vitro* by oat phyA in a light-dependent manner and is also phosphorylated in vivo in response to light (Fankhauser et al., 1999). PKS1 and its homolog PKS2 appear to be involved in regulating the phyA mediated VLFR (Lariguet et al., 2003) although the mode of action is not yet understood.

1.3.4 Phytochrome signalling in the nucleus

More recently, activated phytochromes were found to translocate to the nucleus and to directly interact with transcription factors (Sakamoto and Nagatani, 1996) (Kircher et al., 1999) (Ni et al., 1998), which suggested a surprisingly direct mechanism for light regulation of transcription.

Active phytochromes in their Pfr form interact with a range of transcriptional regulators including members of the Arabidopsis bHLH (basic Helix-Loop-Helix) transcription factor subfamily 15 (Khanna et al., 2004). These include PIF1, PIF3, PIF4, PIF5 and PIF6 (Huq and Quail, 2002; Martinez-Garcia et al., 2000; Ni et al., 1998; Khanna et al., 2004). These proteins bind phyA and/or phyB via the conserved Active-Phytochrome Binding (APB) motif that recognizes only the Pfr form of the phytochrome (Khanna et al., 2004). Other bHLH transcription factors involved in light signalling include SPT, PIL1 and HFR1 (Heisler et al., 2001; Yamashino et al., 2003) (Fairchild et al., 2000). Although they do not bind phyA or phyB directly they can form heterodimers with the PIF bHLH transcription factors.

PIF3 binds directly to the G-box element found in many light regulated promoters, such as from *CCA1*, *LHY*, *RBSC-1A* and *CHS*. Gel supershift assays suggest that PIF3 forms a ternary complex on these promoter sequences that contains phyB in its Pfr form (Martinez-Garcia et al., 2000). Consistent with this, it has been shown that, upon nuclear import, phyB initially forms early speckles in which it colocalizes with PIF3 (Bauer et al., 2004).

The *pif3* mutant has a light hypersensitive phenotype (i.e., a shorter than usual hypocotyl) under continuous irradiation, indicating that it functions as a negative regulator of phytochrome signalling (Bauer et al., 2004; Monte et al., 2004). *pif1* and *pif4* mutants also have a similar phenotype (Huq et al., 2004; Huq and Quail, 2002). PIF3 is rapidly degraded in response to light (Bauer et al., 2004), suggesting that phyB may be binding to PIF3 at the promoters of light regulated genes in order to target it for ubiquitin mediated proteolysis (Park et al., 2004) to allow activation of these light regulated genes.

Mutant and microarray studies have demonstrated that the bZIP transcription factor HY5 (hypocotyl elongated 5) is a major transducer of phytochrome-mediated signalling (Chattopadhyay et al., 1998; Oyama et al., 1997). HY5 can either homodimerize or form a heterodimer with HYH (HY5-homolog) (Holm et al., 2002). Like PIF3 these dimers bind G-box motifs, and because HY5/HYH are positive regulators of phytochrome-mediated signalling they may compete with the repressive PIF3 homo/heterodimers to activate light regulated genes. Also, while PIF3 is degraded in response to light, HY5 shows the opposite behaviour, being present at only very low levels in the dark due to ubiquitin-mediated proteolysis by COP1 (Saijo et al., 2003). In the light COP1-mediated degradation of HY5 is inhibited (Wang et al., 2001) and HY5 levels increase while PIF3 is degraded in a phyB-dependent manner.

Arabidopsis response regulator 4 (ARR4) is a homolog of bacterial response regulators and is a positive regulator of phyB signalling (Sweere et al., 2001). ARR4 is present in both the cytoplasm and the nucleus where it binds the N-terminal tail of phyB (in contrast to most other phytochrome interactors, which bind the C-terminal tail). ARR4 appears to positively regulate phyB activity by stabilizing the Pfr (active) conformer of phyB. To date no signal transduction role has been demonstrated for ARR4, in contrast to bacterial response regulators.

1.3.5 Cryptochromes

The existence of blue-light specific plant responses has been known since the 1800's but in spite of much effort the nature of the proposed blue-light sensing receptor (termed cryptochrome) that was presumed to control these processes remained elusive (Lin, 2002). In 1980 the blue light insensitive *hy4* mutant was described (Koornneef et al., 1980), and cloning of this locus finally showed that it encoded this elusive receptor and that the gene product had high homology to bacterial photolyases (Ahmad and Cashmore, 1993).

Photolyases catalyse the repair of DNA containing cyclo-butyl-pyrimidine or 6-4 photoproducts which are generated by exposure to UV light (Sancar, 1994). This is achieved using the energy from UV-A/blue light which is absorbed by a non-covalently bound folate or deazaflavin and transferred to the catalytic flavin adenine dinucleotide (FAD) chromophore, which then transfers a single electron to the cyclobutane ring of a pyrimidine dimer, causing the cyclobutane ring to collapse and yielding two pyrimidines (Sancar, 2003).

HY4 was renamed *CRY1* (*cryptochrome 1*) and soon after a second cryptochrome was identified and named *CRY2* (Hoffman et al., 1996; Lin et al., 1998). The cryptochromes are slightly larger than photolyases because in addition to the N-terminal PHR (photolyase-related) domain, they also have a small, relatively poorly conserved C-terminal domain that contains a conserved DQXVP-acidic-STAES (DAS) domain that is absent in photolyases (Sancar, 2003). Cryptochromes appear to have arisen from an ancient gene duplication of a *photolyase* gene and are widely distributed in bacteria, animals and plants (Falciatore and Bowler, 2005).

Recently, a third putative cryptochrome was identified in the Arabidopsis genome. This gene, *CRY3,* is a member of the newly defined CRY-DASH (*Drosophila, Arabidopsis, Synechocystis, Homo sapiens*) family (because they were initially identified in the genomes of these four species (Brudler et al., 2003). *CRY3* is only distantly related to *CRY1* and *CRY2* which are thought to originate from the primary endo-symbiont (the ancestral α–protobacterium that gave rise to the mitochondria), because *CRY3* appears to derive from the secondary endo-symbiont (the ancestral cyanobacteria that gave rise to the chloroplast) (Kleine et al., 2003). CRY3 binds FAD and also has DNA binding activity but does not repair DNA. Although it has now become a nuclear-encoded gene, CRY3 is localised in both the chloroplasts and mitochondria (Kleine et al., 2003).

Cryptochromes appear to contain the same folate and FAD chromophores as photolyases (Sancar, 2003) but do not show any DNA repair activity (Ahmad and Cashmore, 1993). While the mechanism of action is not yet clear they appear to function by a similar photon-induced electron transfer mechanism as the photolyases (Lin, 2002; Sancar, 2003).

CRY1 and CRY2 are both nuclear-localised in dark grown plants. In the light CRY1 is exported to the cytoplasm while CRY2 is constitutively nuclear localised (Guo et al., 1999; Kleiner et al., 1999; Yang et al., 2001) but degraded in response to strong light (Lin et al., 1998). In general, these two cryptochromes perform similar functions, with CRY2 mediating the low fluence blue light response and CRY1 mediating the high fluence response. CRY2 is also important for day-length perception and flowering time control.

Recently it has been shown that CRY1 and CRY2 become phosphorylated in response to blue light (Shalitin et al., 2002; Shalitin et al., 2003). This appears to be by autophosphorylation, in spite of no obvious kinase motif within the protein. Furthermore, phosphorylation is necessary for light signalling activity. CRY1 and CRY2 both form homodimers (Sang et al., 2005), and this interaction is mediated by the N-terminal PHR domain. Based on these data it has been proposed that blue light drives a conformational change in the PHR domains of the homodimer that brings the two C-terminal domains together, which then autophosphorylate and become active (Sang et al., 2005).

The C-terminal domains of CRY1 and CRY2 bind the E3 ubiquitin ligase COP1 in both light and dark grown plants, and blue light activation of CRY1/CRY2 inhibits the ligase activity of COP1 (Wang et al., 2001; Yang et al., 2001). Overexpression of a dominant-positive CRY C-terminal domain results in a constitutive photomorphogenic (COP) phenotype in dark grown plants, suggesting that CRY inhibition of COP1 is a key step in the de-etiolation process.

Many studies using mutant *cry* and *phy* alleles have shown that phytochrome and cryptochrome signalling are highly synergistic (i.e., cryptochromes require phytochromes, and *vice versa*) (Ahmad and Cashmore, 1997; Devlin and Kay, 2000; Neff and Chory, 1998). Experiments showing direct interactions between phyA and CRY1 (Ahmad et al., 1998), as well as between phyB and CRY2 (Mas et al., 2000) suggest that they may directly activate each other or otherwise modulate each others activity. Alternatively the calcium binding protein SUB1 has been proposed to act as an integration point for phytochrome and cryptochrome signals (Guo et al., 2001).

1.3.6 Phototropins

Plants sense not only the quantity and quality of light they receive, but also the direction from which it is coming (Lin, 2002). Plants respond by bending of the

hypocotyl so that they grow towards the direction of light, and this response is known as positive phototropisim. In contrast, roots grow away from light (negative phototropisim). It has been known for over a century that this phototropic response is mediated exclusively by blue light in most plant species (Lin, 2002), but analysis of *cry1,cry2* mutants showed they had wild-type phototropic responses, suggesting that another blue-light photoreceptor was involved.

A screen for phototropic mutants yielded the *nph* (non-phototrophic hypocotyl) mutants with impaired hypocotyl and root curvature in response to blue light (Liscum and Briggs, 1995). The *NPH1* locus was cloned (Huala et al., 1997) and shown to encode a protein of approximately 120 kDa with two N-terminal LOV (light oxygen voltage) domains and a C-terminal kinase domain. This locus has now been renamed *PHOT1* (*phototropin1*). The *phot1* mutant still retains phototropic responses under high fluence light, suggesting the existence of an additional photoreceptor that mediates the high fluence response. Subsequently, a mutant which abolished the high-fluence phototropic response was isolated and named *npl1* (*nph1*-like). The *NPL1* locus was found to encode a *PHOT1* homolog which was named *PHOT2* (Jarillo et al., 2001b; Sakai et al., 2001). Further studies confirmed that phot1 mediates the low fluence phototropic response and is unstable under high fluence blue light, while phot2 is responsible for the high fluence response, a situation which is remarkably similar to that for CRY1 and CRY2 (Sakai et al., 2001).

Recent studies have shown that the two N-terminal LOV (light oxygen voltage) domains are responsible for chromophore binding and that each can non-covalently bind a flavin mononucleotide chromophore (Christie et al., 1999). It appears that absorption of blue light by the chromophore activates a C-terminal serine/threonine kinase activity which autophosporylates itself and possibly also other substrates (Christie et al., 1998). Like phytochromes and cryptochromes, phototropins also form dimers, with the LOV1 domain apparently mediating dimerization (Salomon et al., 2004).

phot1 (and presumably also phot2) are localised to the plasma membrane in the dark (Sakamoto and Briggs, 2002). Because phototropins lack any obvious membrane spanning domains it appears that the highly hydrophilic nature of these proteins is sufficient to drive this localisation. In blue light phot1 appears to dissociate from the membrane (possibly as a result of the autophosphorylation) and to localise to the cytoplasm and possibly also the nucleus (Sakamoto and Briggs, 2002).

In contrast to phytochrome and cryptochrome mutants, phototropin mutants display no major photomorphogenesis or flowering time defects. This indicates that they act in largely separate pathways from the other photoreceptors and do not play a major role in light-regulated plant development. In addition to regulation of the directional tropic responses (Liscum and Briggs, 1995) they are also involved in controlling chloroplast movement. For example, in low light phototropins are necessary for ensuring that the chloroplasts do not shade each other, thus maximising photosynthetic efficiency, while under high-fluence conditions phototropins mediate chloroplast shading so as to reduce the amount of light they are exposed to and to minimize photodamage to the photosynthetic apparatus (Jarillo et al., 2001b; Kagawa and Wada, 2002; Sakai et al., 2001). In addition to their role in phototropism and chloroplast localisation phot1 and phot2 also mediate blue-light induced opening of the stomata (Kinoshita et al., 2001).

The *nph3* mutant was recovered in the same screen as the one that identified *phot1*, and the *nph3* and *phot1* mutants both have the same phenotypes (Liscum and Briggs, 1996). Mapping of the mutation showed that the *NPH3* locus encodes a BTB/POZ and coiled-coil domain containing protein. The *rpt2* mutant also has a similar phenotype and encodes an *NPH3* homolog with BTB/POZ and coiled-coil domains (Sakai et al., 2000). NPH3 and RPT2 interact with each other and also bind the N-terminus of phot1 (Motchoulski and Liscum, 1999; Inada et al., 2004). NPH3 may also be phosphorylated by phot1 in response to blue light while RPT2 is necessary for phot mediated opening of stomata (Motchoulski and Liscum, 1999) (Inada et al., 2004).

The *nph4* mutant was identified in the same screen as *phot1 and NPH3*, but in addition to showing impaired phototropisim it also has impaired gravitropisim (Liscum and Briggs, 1996; Stowe-Evans et al., 1998). Mapping showed that *nph4* contained a mutation in the *ARF7* (auxin-response-factor 7) locus (Harper et al., 2000), suggesting a mechanism by which phototropins may regulate transcriptional regulation.

1.3.7 Novel photoreceptors

In addition to the classical photoreceptors, Arabidopsis contains a small gene family consisting of *ZTL* (Zeitlupe), *LKP2* (LOV Kelch protein 2) and *FKF1* (Flavin-binding Kelch repeat F-box 1) (Jarillo et al., 2001a; Nelson et al., 2000; Schultz et al., 2001;

Somers et al., 2000). The encoded proteins each consist of an N-terminal LOV domain similar to those found in phototropin, an F-Box domain (typically involved in ubiquitin-mediated protein degradation), and a series of C-terminal Kelch repeats (typically involved in mediating protein-protein interactions). Like phototropin the LOV domain binds FMN (flavin mononucleotide) and absorbs blue light, suggesting they are bona fide photoreceptors (Cheng et al., 2003; Imaizumi et al., 2003). ZTL family members act as substrate adaptors for a Cullin1/SCF-based ubiquitin ligase complex (Han et al., 2004).

ztl mutants and lines overexpressing *LKP2* both show misregulation of the circadian clock (Jarillo et al., 2001a) (Schultz et al., 2001), suggesting that they target components of the circadian clock for ubiquitin-mediated proteolysis in a light-dependent manner. ZTL has been shown to bind directly to and mediate the degradation of TOC1, a core component of the circadian clock central oscillator (Mas et al., 2003). ZTL has also been shown to bind CRY2 and phyB directly (Jarillo et al., 2001a). In contrast to *ZTL* and *LKP2*, expression of *FKF1* is itself regulated by the circadian clock and FKF1 appears to function as a sensor for long day growth conditions (which in *Arabidopsis* and other "long day" plants triggers flowering). Circadian regulated transcription ensures that FKF1 is only present in the early evening, and so it can only be activated by light during long day conditions (Imaizumi et al., 2003). Activated FKF1 acts as a substrate adaptor for a ubiquitin ligase that mediates the proteolysis of the cycling-DOF repressors of flowering. Degradation of the cycling-DOF factors relieves repression of the *CONSTANS* gene, the key regulator of the vegetative to floral transition (Imaizumi et al., 2005).

1.3.8 UVB light perception

High fluence UVB light is damaging to all organisms, and plants have developed stress-response mechanisms to avoid/repair UV-induced DNA damage. Low fluence UVB light can induce photomorphogenic responses in plants, such as inhibition of hypocotyl elongation (Kim et al., 1998; Suesslin and Frohnmeyer, 2003). These responses do not appear to be mediated by the phytochrome/cryptochrome/phototropin photoreceptors, but in spite of numerous attempts no specific UVB photoreceptor has yet been identified (Ulm and Nagy, 2005). However recent work has shown that the bZIP transcription factor HY5 is critical for mediating UV-B induced changes in gene expression (Ulm et al., 2004) in addition to its central role in visible light signalling pathways.

A UVB sensitive mutant *uvr8*, (Kliebenstein et al., 2002) was cloned and found to encode a plant homolog of *RCC1* (regulator of chromatin-condensation 1), a chromatin-binding protein that is localised to the nuclear pores. *HY5* is down regulated in the *uvr8* background and UVR8 binds directly to the *HY5* promoter (Brown et al., 2005). These results suggest that UV-B light perception may function via UVR8 mediated activation of *HY5*.

1.4 cop/det/fus mutants

1.4.1 Introduction

Another group of genes involved in light signalling were recovered in screens for mutations that cause photomorphogenic development to commence spontaneously even in complete darkness. These mutants known as the *cop (constitutive photomorphogenesis)/det (de-etiolated)/fus (fusca)* mutants (the last group were recovered in a screen for excessive pigment accumulation in seeds and were found to be epistatic to the *cop/det* mutants) (Castle and Meinke, 1994; Deng et al., 1991; Misera et al., 1994; Chory et al., 1989). As well as the *cop/det* phenotype, other abnormalities include chloroplast development in the roots, excessive anthocyanin production, excessive root branching and reduced apical dominance, day-length insensitive early flowering and partial sterility (Pepper and Chory, 1997). These phenotypes are displayed in partial-loss-of-function mutations, with complete loss of function alleles been seedling lethal. When grown in the light the *cop/det/fus* mutants display light hypersensitive phenotypes. Because this contrasts with the *hy* mutants described earlier, it was proposed that *COP/DET/FUS* gene products are negative regulators of light signalling.

Microarray analysis show that the gene expression patterns of dark grown *cop/det/fus* mutants are almost identical to those of light-grown wild type plants (Ma et al., 2003) confirming that the observed phenotype is indeed due to premature initiation of the light-grown (de-etiolated/photomorphogenic) development programme.

Cloning and characterisation of the *COP/DET/FUS* loci revealed that most of them form part of an 8-subunit multi-protein complex called the CSN (COP9 signalosome; (Chamovitz et al., 1996), which appears to be conserved in almost all eukaryotes (Schwechheimer, 2004). COP1, COP10 and DET1 are also found in multiprotein

complexes involved in the same pathway(s) as the CSN in plants and animals, but are less well conserved among other eukaryotic taxa.

1.4.2 COP9 Signalosome

When the COP9 protein was originally purified (Chamovitz et al., 1996) it was found to be part of a complex that also contained the protein products of the genes mutated in the *cop8*, *cop11*, *fus5*, *fus11* and *fus12* mutants (Schwechheimer, 2004). This complex, termed the CSN (COP9-signalosome) is evolutionarily related to the 19S lid of the 26S proteasome which degrades ubiquitinated proteins (Hershko and Ciechanover, 1998), suggesting that the CSN may also be involved in ubiquitin-mediated proteolysis. The proteins that make up the CSN have since been renamed CSN1-8 (Deng et al., 2000). Most of these proteins have a conserved PCI (Proteasome, COP9 and eIF3) domain which is believed to be involved in assembling the complex (Hofmann and Bucher, 1998). CSN5 also has a JAMM metalloproteinase domain which is essential for CSN activity (Cope et al., 2002). The signalosome was subsequently found to interact directly with the cullin subunit of cullin-based E3 ubiquitin ligases (Lyapina et al., 2001).

1.4.3 Ubiquitin-Proteasome System

Protein abundance in the cell is controlled not only by the rate of transcription and translation but also by the rate at which proteins are degraded. The rate of degradation varies greatly for different proteins and can also vary depending on the prevailing cellular conditions (e.g., stage of the cell cycle, cell type etc.). Protein degradation is performed by the ubiquitin-proteasome system which consists of a series of enzymes that conjugate chains of the ubiquitin tag (a small 76 amino acid protein) to the target protein. Ubiquitin is activated by the E1-activating enzyme which binds C-terminal glycine on ubiquitin via an ATP-dependent thioester to the active-site cysteine of the E1-activating enzyme. The activated ubiquitin is then transferred to the active-site cysteine of the E2-activating enzyme. The E2-activating enzyme is recruited by an E3-ligase which also binds the target protein and catalyses the transfer of the activated ubiquitin from the E2-activating enzyme to a lysine residue on the target protein. Some E3-ligases such as Hect-domain family members also have an active-site cysteine to which the activated ubiquitin is transferred prior to being ligated to the target protein. In other cases the E3 ligase acts to bring the target and the E2-ubiquitin together and thus achieve target specificity. This process is

repeated with additional ubiquitin moieties added to the first one to form a ubiquitin "chain" (Fig 1.1).

Protein degradation is performed by the 26S proteasome, a large complex consisting of the barrel shaped 20S subunit flanked by a 19S "lid" subunit at either end of the barrel. The 19S lids recognize the ubiquitin chain(s) on the target protein and feed the target protein into the 20S barrel where the peptide bonds are cleaved to yield free peptides and short peptide chains.
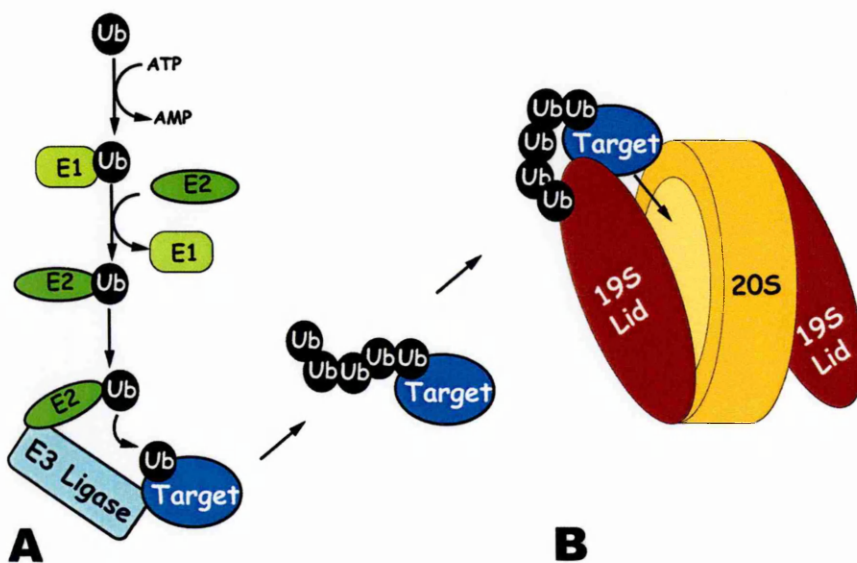
Fig 1.1 Ubiquitin mediated degradation pathway **(A)** Ubiquitin is conjugated to the E1 ubiquitin ligase enzyme in an ATP dependent manner. The ubiquitin moiety is then transferred to an E2 ubiquitin ligase enzyme. An E3 ubiquitin ligase binds both the E2 ligase and the target protein to be ubiquitinated and the ubiquitin moiety is transferred to a lysine residue on the target protein. This cycle can be repeated a number of times with each additional ubiquitin moiety being transferred to a lysine residue on a ubiquitin moiety already conjugated to the target protein, forming a polyubiquitin chain. **(B)** The 26S proteasome consists of the barrel shaped 20S catalytic core with two 19S lids. The polyubiquitin chain on the target protein is recognized by the 19S lid which feeds the target protein into the 20S catalytic core where it is degraded.

1.4.4 Cullin-based ubiquitin ligases

Cullin-based E3 ubiquitin ligases are multisubunit complexes which poly-ubiquitinate target proteins, targeting them for degradation by the 26S proteasome (Schwechheimer and Villalobos, 2004). The cullin acts as a scaffold protein that binds a substrate adaptor, which is of a different type for each Cullin. The substrate

adaptor for Cul1 consists of SKP and an F-box containing protein that directly binds and holds the substrate (the protein to be ubiquitinated) in position while the other end of the cullin binds RBX, which in turn recruits a ubiquitin E2 ligase conjugated to ubiquitin. The complex catalyses the transfer of the ubiquitin moiety from the E2 directly to the substrate protein (Moon et al., 2004).
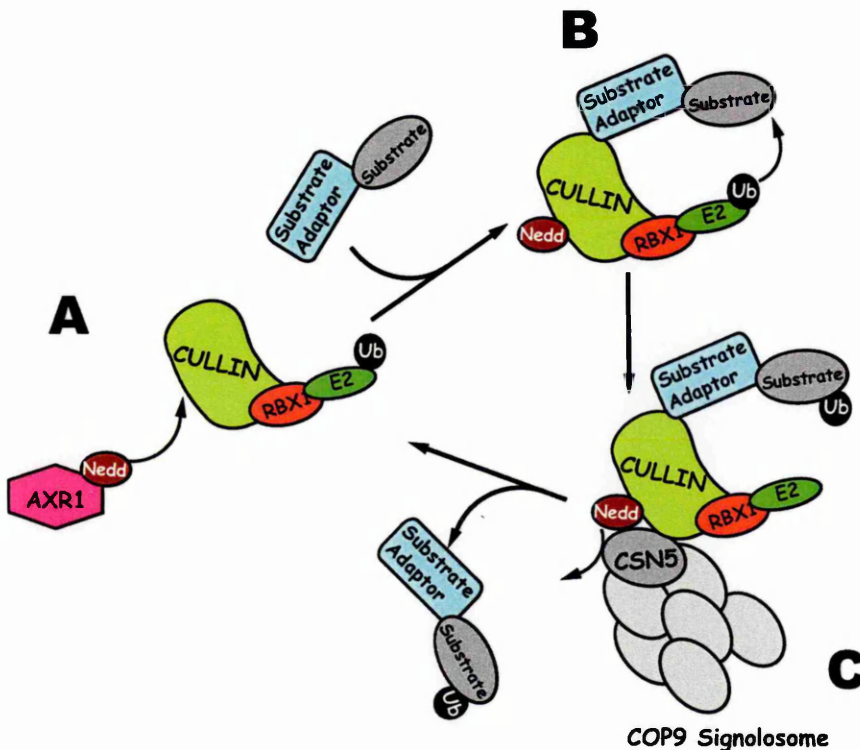
Fig 1.2 Regulation of Cullin-based Ubiquitin ligases **(A)** AXR1 conjugates the ubiquitin like Nedd8 protein to the Cullin subunit of an E3 ligase complex, this favours the binding of a substrate adaptor to the cullin. **(B)** The E3 ligase complex catalyses the transfer of the ubiquitin moiety to the substrate protein. **(C)** The CSN5 subunit of the COP9 signalosome cleaves Nedd8 from the cullin, favouring the dissociation of the substrate adaptor.

Plants have at least 3 distinct cullins (Schwechheimer and Villalobos, 2004) and about 700 different F-box domain-containing proteins, each of which is likely to be specific for a different target protein (Risseeuw et al., 2003), suggesting that Cullin E3 ligases are major regulators of protein abundance in plants (Thomann et al., 2005).

Cullin itself is subject to post-translational modification by conjugation to a small protein RUB1/Nedd8, which is related to ubiquitin. Neddylation of Cullin, catalysed by ARX1 (del Pozo et al., 2002), favours the interaction of Cullin with the SKP1/F-box substrate adaptor, while de-Neddylation (by the JAMM metalloproteinase of CSN5 from the COP9 signalosome; (Cope et al., 2002) favours dissociation of the substrate adaptor SKP1/F-box. Cullin is continuously neddylated and deneddylated in a cyclic fashion, and this is apparently necessary for the function and stability of the cullin complex (Cope and Deshaies, 2006) Fig 1.2).

Mutations in components of the csn subunits abolish CSN activity and result in only neddylated cullins being present. These neddylated cullins have impaired E3 ubiquitin ligase activity (possibly because cyclic binding and dissociation from the substrate adaptor is necessary for correct function, or because excessive neddylation of the cullin results in it ubiquitinating and degrading the substrate adaptor itself (Cope and Deshaies, 2006), thus impairing their ability to ubiquitinate the target proteins. The cop phenotype in the csn mutants appears to be due to their inability to degrade positive regulators of light signalling when the plants are grown in the dark. This leads to an increase of positive regulators such as HY5, which eventually trigger the initiation of the light induced developmental cascade.

1.4.5 cop1

Cloning of the COP1 locus showed that it encodes a protein containing an N-terminal RING domain (a domain which often has E3 ubiquitin ligase activity), a coiled-coil domain (a multimerization/protein-protein interaction domain) and 7 WD40 repeats (protein-protein interaction domains) (Deng et al., 1992). COP1 homodimerizes and forms part of a 700 kDa complex that is localised to distinct nuclear speckles in the dark and to the cytoplasm in the light (Torii et al., 1998; von Arnim et al., 1997) (Saijo et al., 2003) .

COP1 has been shown to have ubiquitin ligase activity against a number of positive regulators of light signalling, such as HY5, HYH, LAF1 and HFR1 in dark grown plants, while in the light it degrades two light-labile photoreceptors; CRY2 and phyA (Holm et al., 2002; Saijo et al., 2003; Seo et al., 2004; Seo et al., 2003) (Duek et al., 2004). COP1 also interacts with but does not degrade CRY1 and phyB. While little is known about the nature of the COP1-phyB interaction, it appears that the CRY1 homodimer is constitutively bound to the COP1 homodimer (presumably as part of

the 700 kDa complex) (Sang et al., 2005). When CRY1 absorbs blue light a conformational change occurs in the C-terminal (COP1-interacting) domain, which inactivates the E3 ligase activity of COP1 (Wang et al., 2001).

1.4.6 *det1*

The *det1* mutant has a de-etiolated phenotype when germinated in the dark. Other phenotypic abnormalities include excessive anthocyanin accumulation, chloroplast development and greening of the roots, reduced apical dominance, day-length insensitive early flowering, abnormal flower development (resulting in partial male sterility) and aberrant leaf morphology (Chory et al., 1989; Chory and Peto, 1990; Pepper et al., 1994; Pepper and Chory, 1997). The *det1* mutant also shows aberrant regulation of many light regulated genes such as *CAB*, *CHS*, *RBCS* and *PSAA/PSAB* (Chory and Peto, 1990). Map based cloning of the *DET1* gene identified a 62 kDa protein with no recognizable domains (Pepper et al., 1994). The DET1 protein was shown to be constitutively nuclear localised but does not bind DNA (Pepper et al., 1994). Further studies showed that DET1 forms part of a complex with the plant homologue of DDB1 (damaged-DNA-binding protein1), a protein involved in repair of damaged DNA in mammals (Schroeder et al., 2002).

1.4.7 *det1/ddb1a* enhanced phenotype

Arabidopsis has two DDB1 homologs, DDB1A and DDB1B. While the *ddb1b* mutant appears to be embryo lethal, the *ddb1a* mutant has no phenotype on its own. When *ddb1a* was crossed into the medium-strength *det1-1* allele, the resulting *ddb1a/det1-1* double mutant showed enhancement of the *det1* phenotype. In particular, the plants had even shorter hypocotyls, higher anthocyanin content compared to the *det1-1* single mutant and were completely sterile (Schroeder et al., 2002).

1.4.8 The CDD complex

Cloning of the *cop10* locus showed that it encoded a 16 kDa protein with homology to ubiquitin E2 ligases (Suzuki et al., 2002). However, COP10 lacks the conserved cysteine residue found in all E2 ligases that is required for ubiquitin conjugation. Instead COP10 appears to belong to the ubiquitin E2 variant (UEV) family which form heterodimers with bona-fide E2 enzymes and enhance their activity (Sancho et al., 1998). This was indeed found to be the case for COP10, which increased the ubiquitination activity of a number of E2 enzymes (Yanagawa et al., 2004).

COP10 was shown to be part of a 300 kDa complex that also contains DET1 and DDB1A, termed the CDD (COP10 DET1 DDB1) complex (Yanagawa et al., 2004). COP10 is necessary for COP1-mediated degradation of HY5 (Osterlund et al., 2000) and COP10 can also bind directly to COP1 (Suzuki et al., 2002). From gel filtration studies it is clear that COP10 and COP1 are in two separate complexes of 300 kDa and 700 kDa, respectively (Saijo et al., 2003; Yanagawa et al., 2004). Instead it is likely that the CDD complex binds the COP1 complex and modulates its activity, possibly by recruitment of an activating E2 ubiquitin ligase.

1.5 Tomato hp mutants

A number of mutants with aberrant light responses have also been identified in tomato (*Solanum lycopersicum*). Of particular interest are the light hypersensitive *hp* (*high pigment*) mutants (Kendrick et al., 1994). *hp1* was isolated as a spontaneously occurring mutation at the Campbell Soup Company farms (Riverton NJ) in 1917, while the *hp2* mutant was reported in the Italian San Marzano variety (Kendrick et al., 1994). Both of these mutations are light hyper-responsive, with elevated levels of anthocyanin, shorter hypocotyls and increased flavonoid and carotenoid levels in the fruit (Mustilli et al., 1999).

1.5.1 *hp2*

The *HP2* locus was mapped and found to encode the tomato homologue of DET1. tDET1 (tomato DET1) shares 75% identity with *Arabidopsis* DET1 and is present as a single copy gene in tomato (Mustilli et al., 1999). Unlike *det1*, the *hp2* mutant (which has a splice site mutation resulting in only 10% wild type transcript) does not have noticeable de-etiolated phenotypes when grown in the dark, which lead to a suggestion that DET1 function was different in the two different species. However analysis of the stronger tomato *hp2^j* mutant (with a single amino acid substitution) showed that it has some de-etiolated phenotypes in the dark, such as plastid development (Mustilli et al., 1999). While the phenotypes of the tomato *hp* mutants are generally not as strong as those of the *det1* mutants in *Arabidopsis*, this appears to be simply because they are weaker mutants.

1.5.2 *hp1*

The *HP1* locus was mapped and found to encode the tomato homologue of DDB1. tDDB1 (which appears to be a single copy gene in tomato) shares 86% identity with *Arabidopsis* DDB1A (Liu et al., 2004). The *hp1* and stronger *hp1^w* mutants both

contain single point mutations, resulting in a single amino-acid substitution, and are probably only partial loss of function mutants.

1.5.3 Genetic interaction between *hp1* and *hp2*

The *hp1 hp2* double mutant shows a stronger phenotype than the individual single mutants, in particular when the stronger $hp2^j$ and $hp1^w$ alleles are used (Liu et al., 2004), because the double mutant is homozygous lethal. Given that all these alleles are only partial loss of function mutants, the additive phenotype indicates that the two genes may or may not act in the same pathway and is consistent with the interaction between DET1 and DDB1A demonstrated at the protein level in *Arabidopsis* (Schroeder et al., 2002).

1.6 DET binds chromatin

DET1 had been shown to be a negative regulator of light-induced gene expression (Chory et al., 1989; Chory and Peto, 1990) and while the DET1 protein localises to the nucleus, it shows no DNA binding activity (Mustilli et al., 1999; Pepper et al., 1994). The DET1 protein also has no identifiable domain or motifs that could give a clue to its mode of action. The only possible clue came from the studies of the *Drosophila ABO1* (Abnormal oocyte 1) gene. The *abo1* mutants display a maternal-specific embryogenesis defect (Tomkiel et al., 1995). ABO1 shares 25% identity with the Arabidopsis DET1 protein (Berloco et al., 2001), and was shown to bind the core histone gene promoters during early embryogenesis to downregulate expression of these genes (Berloco et al., 2001).

Tomato DET1 binds mononucleosomes *in vitro*, and this interaction was shown to be mediated via a specific interaction with the N-terminal tail of the core histone H2B. Acetylation of the N-terminal tail also reduced binding of DET1 (Benvenuto et al., 2002). FRET (fluorescence resonance energy transfer) experiments on plant cells expressing H2B and DET1 tagged with ECFP and EYFP (enhanced cyan fluorescent protein and enhanced yellow fluorescent protein), respectively, showed that they also interact *in vivo* (Benvenuto et al., 2002).

1.7 Role of DDB1 in DNA damage repair

DDB1 was first characterized in mammalian systems, where it is an essential component of a complex involved in DNA damage repair (Wittschieben and Wood, 2003). In mammals a variety of DNA lesions including UV induced cyclobutane

pyrimidine dimers (CPD's) and 6-4 photoproducts are repaired by the Nucleotide Excision Repair (NER) pathway. In NER, damaged nucleotides such as 6-4 photoproducts are individually excised from the DNA and replaced with a new nucleotide using the opposing strand as a template (Tang and Chu, 2002).

Mutation of genes coding for proteins involved in the NER pathway results in a genetic disorder known as Xeroderma Pigmentosum (XP), which is characterised by hypersensitivity to sunlight and a high susceptibility to UV-induced skin cancers.
7 different sub-types, or complementation groups, of XP have been identified, each one being mutated at different loci encoding components of the DNA damage repair machinery (Wittschieben and Wood, 2003).

The XP-E complementation group was originally defined as lacking a factor in the crude cell extracts that bound UV-irradiated DNA. This complex was named the UV-DDB (UV-Damaged DNA Binding) complex. Two proteins present in the complex were identified and named DDB1 and DDB2, and sequencing revealed that all members of the XP-E complementation group had mutations in the *DDB2* gene (Wittschieben and Wood, 2003).

Further studies have shown that DDB1/DDB2 are part of a large complex that includes Cul4 and Roc1, that is activated in response to UV irradiation and then recognizes and binds directly to damaged DNA (Groisman et al., 2003). This DDB1 complex does not appear to have any DNA repair activity itself, rather it serves to recognize the site of damage (Wittschieben et al., 2005) and recruit the DNA repair complexes such as XPC. The activated DDB1 complex also poly-ubiquitinates itself and its interacting partners (Sugasawa et al., 2005). Self-ubiquitination causes the DDB1 complex to dissociate from the DNA, making space for other components of the DNA repair machinery. Ubiquitination of its interacting partners may increase their DNA binding affinity (Sugasawa et al., 2005). Recently it has been shown that DDB2 (and thus presumably the whole Cul4/DDB1/DDB2 complex) is required for re-establishment of ubiquitinated histone H2A after the repair of damaged DNA (Kapetanaki et al., 2006).

In addition to it's role in DNA damage repair, a complex consisting of Cul4/DDB1/hDET1/hCOP1 was shown to ubiquitinate and degrade c-Jun, a mammalian homolog of HY5 (Wertz et al., 2004). Human COP1 (hCOP1) has also

been shown to ubiquitinate and degrade p53, a key cell-cycle regulator (Dornan et al., 2004).

1.8 Histone code

Eukaryotic DNA is incorporated within chromatin, a polymer which contains histone and non-histone proteins (Kornberg, 1977) The core unit of chromatin is the nucleosome, which consists of 1 and 3/4 turns of double-stranded DNA wrapped around an octamer of histone proteins (two each of H2A, H2B H3 and H4), (Luger et al., 1997). Nucleosomes in turn are organised into higher order structures (Olins and Olins, 1974), which serve to compact the DNA so that it is compact enough to fit in the nucleus and to organize it (Wolffe and Hayes, 1999). From microscopic studies it has long been known that different regions of chromatin exist within the nucleus, with the dense and gene poor heterochromatin consisting of mainly non-coding and repetitive sequences while gene rich regions are mainly found within the de-condensed euchromatin regions.

Chromatin itself is subject to a vast array of post-translational modifications, with most of these occurring on the histone N-terminal tails, which extend out from the nucleosome. These modifications include methylation, acetylation phosphorylation ADP-ribosylation, ubiquitination and SUMOylation (Turner, 2002), while DNA is subject to methylation on cytosine residues. Heterochromatin is generally devoid of acetylation and is enriched in histone H3 methylated at lysine's 9 and 27 (H3K9me and H3K27me) and histone H4 methylated at lysine 20 (H4K20me). Euchromatin is less dense, is gene rich, and contains mainly transcribed DNA sequences. The tails of histones within euchromatin are often acetylated, while histone H3 is often methylated at lysine's 4 and 36 (H3K4me and H3K36me) (Fischle et al., 2003). These marks appear able to selectively recruit a wide variety of proteins which can act as signals to add more modifications to the chromatin to modify nucleosome spacing along the DNA, to activate or repress transcription (Bannister et al., 2001; Kanno et al., 2004; Wysocka et al., 2005). In addition histone acetylation reduces the net positive charge of the histones and may make it easier for the transcriptional apparatus to remove or move through them (Wolffe and Hayes, 1999). These post-translational chromatin modifications affect the level of transcription of the DNA that they are associated with, and as such this represents an additional level of information that is encoded within the chromatin but is at least semi-independent of DNA sequence. Based on the vast combinatorial possibilities of different modifications that can be present together on a nucleosome, it has been proposed

that these marks are read by specific proteins as a "histone code" analogous to the genetic code contained within the DNA (Jenuwein and Allis, 2001).

It is now well established that this "histone code" acts to regulate the transcription of the genes and other chromatin processes (Nightingale et al., 2006). One role of these chromatin modifications may be to reduce noise in gene expression by locking in transcriptional states and achieving more stable, regulated transcription. An important feature of this "histone code" is that, like the DNA sequence itself, chromatin modifications are propagated and replicated through mitosis. This means that it can act as a form of "cellular memory" that propagates information on gene expression levels of various genes throughout successive cell divisions. This is particularly important in muilticellular organisms, which contain many different cell types, each with their own cell-type specific gene expression patterns that all contain the same DNA or "genetic code." Cell-type specific gene expression profiles are specified by cell-type specific expression of transcriptional regulators such as HOX transcription factors and then maintained through subsequent rounds of cell division by the "histone code" which locks in and propagates these gene expression profiles even in the absence of the original signal (the HOX factor) (Levine et al., 2004). Of particular importance in this process are the polycomb and trithorax protein complexes which act to lock in repression or activation of their targets, respectively (Levine et al., 2004) and are conserved throughout muilticellular eukaryotes.

In contrast to the relatively fixed developmental pattern of animals, plant development is controlled to a large extent by their external environment. Mutagenesis screens for plants with altered development or environmental responses have recovered many loci encoding proteins involved in chromatin remodelling and modification. This suggests that plants sense their environment and then adjust their development in response to these signals by remodelling chromatin in order to achieve the required gene expression patterns. Among the best studied examples of this is the vegetative-to-floral transition, a key developmental transition governed by many environmental inputs such as light quality, temperature, day-length and exposure to cold (Boss et al., 2004). These inputs act via chromatin remodelling proteins to alter the expression of key regulators controlling the vegetative-to-floral transition. For example, cold induces the down-regulation of the key negative regulator of flowering *FLC*, a process which is essential for flowering in annual Arabidopsis ecotypes. This is achieved by recruiting histone deacetylases and demethylases (VIN3, FLD) to remove activating marks, followed by recruitment

of histone methyltransferases such as VRN1 and VRN2, that act to lock in the silencing of this gene once the plant returns to warmer conditions, e.g., in Spring (He and Amasino, 2005).

In contrast to *FLC*, *FT* is a key positive regulator of flowering and is repressed by LHP1 (like heterochromatin protein 1) during early development. LHP1 binds repressive chromatin modifications such as H3K9me and acts to recruit histone methyltransferases and other histone modifying proteins so as to lock in the silent state. This repression is antagonized by *CONSTANS*, a circadian regulated gene which is only expressed under long day conditions and activates *FT*, a key promoter of flowering (Ausin et al., 2005).

De-etiolation, the transition from skotomorphogenic to photomorphogenic growth, is another major developmental transition induced by environmental stimuli, in this case by light, which is sensed by the photoreceptors and that results in a major reprogramming of gene expression (Ma et al., 2001). While most loci found to be involved in de-etiolation have been found to encode photoreceptors, transcription factors or components of the ubiquitin-mediated proteolysis pathway, the recovery of a chromatin binding protein DET1 and its interacting partners DDB1 and COP10, suggests a role for chromatin remodelling in the de-etiolation transition, especially considering the large number of loci whose gene expression profile is modulated by light signals (Ma et al., 2001). In particular, it has been hypothesized that the DET1 complex interacts with the chromatin around light-activated target genes to maintain them in a repressed state in darkness (Benvenuto et al., 2002).

1.9 Thesis Aim

The aim of this study was to investigate the putative role of chromatin remodelling in photomorphogenesis and in particular to investigate the hypothesis that the DET1 complex interacts with the chromatin around light-activated target genes to maintain them in a repressed state in darkness (Benvenuto et al., 2002). In order to test this hypothesis the aims of this project were to 1: Identify the genomic targets of the DET1 complex (using chromatin immunoprecipitation) and characterize these interactions, 2: Investigate the function of the DET1 complex at its target sites and the mechanism(s) by which it is recruited. In particular, the role of chromatin structure and modification (the histone code; (Fischle et al., 2003) in these processes has

been examined. 3: Investigate global changes in chromatin modifications and structure that occur during photomorphogenesis.

2. Materials and methods

2.1 Materials

2.1.1 Chemicals, solvents and Reagents

Unless otherwise specified all reagents are analytical or cell biology grade and made up in double-distilled water (DDW).

Sigma-Aldrich (St. Louis, MO): 17-β-estrodiol, ampicillin (sodium salt), basta (DL-Phosphinothricin), β-mercaptoethanol, bovine serum albumin (BSA), DAPI (4',6-diamidino-2-phenylindole) hygromycin-B, kanamycin (sodium salt), N,N,N,N-tetramethylethylenediamine (TEMED) NP40 detergent, paraformaldehyde, sodium-deoxycholate, spectinomycin (sodium salt), Tween20 detergent, Triton-X 100 detergent,

GE healthcare (Little Chalfont, UK): Enhance chemiluminescence agent, Protein-A sepharose beads

Biorad (Hercules, CA): Acrylamide

JT Barker (Phillipsburg, NJ): Formaldehyde, ethanol, methanol

Proddoti Gianni (Milan, Italy): Agarose (molecular biology grade)

Leahe Seeds (Round Rock, TX): Silwet L77 surfactant

Roche (Basel, Switzerland): Complete EDTA-free protease inhibitor tablets, AEBSF

Upstate (Lake Placid, NY): Protein-A agarose

Vector Laboratories (Cambridge, UK): Vectorshield H-1000

Invitrogen (Carlsbad, CA): Murashige and Skoog (MS) Basal Medium

Duchefa (Haarlem, Holland): B5 vitamins

All other standard reagents were obtained from the following suppliers: Sigma-Aldrich (St. Louis, MO), JT Barker (Phillipsburg, NJ), Proddoti Gianni (Milan, Italy), Promega (Madison WI) or Boehringer Mannheim (Amsterdam, Netherlands).

2.1.2 Enzymes

Taq DNA polymerase (EC 2.7.7.7)	**BioGem** (Naples, Italy)
High Fidelity DNA polymerase (EC 2.7.7.7)	**Roche** (Basel, Switzerland)
LR DNA Recombinase	**Invitrogen** (Carlsbad, CA)
Proteinase K, RNAaseA	**Sigma-Aldrich** (St. Louis, MO)
T4 DNA ligase (EC.6.5.1.1)	**Promega** (Madison WI)
Cellulose, Macerozyme	**All Japan Biochemicals Co.** (Nishinomiya, Japan)

Type II restriction endonucleases (EC 3.1.21.4)	
*Bam*H I	**GE healthcare** (Little Chalfont, UK)
Xho I *Not* I, *Pst* I	**New England Biolabs** (Ipswich, MA)
Sal I,	**Promega** (Madison WI)

2.1.3 Antibodies

Abcam (Cambridge, UK): rabbit anti-MYC (ab9106), rabbit anti-GFP (ab6556)

Molecular Probes (Eugene, OR): Alexaflour 488-conjugated goat anti-rabbit

Pascal Genchick (Strasbourg, France): rabbit anti-Cul4

Pierce biochemicals (Rockford, IL): Horseradish peroxidase (HRP)-conjugated goat anti-rabbit, HRP-conjugated goat anti-mouse

Roche (Basel, Switzerland): mouse Anti-HA (12C5, monoclonal)

Upstate (Lake Placid, NY): rabbit anti-H3K4me2 (#07-030), rabbit anti-H3K9me2 (#07-441) mouse anti-MYC (4A6, monoclonal)

2.1.4 Oligonucleotides

Single strand oligonucleotide primers for or semi-quantitative PCR analysis and DNA fragment synthesis by polymerase chain reaction (PCR) amplification or annealing of double stranded fragments were obtained from Primm (Milan, Italy), see Appendix A for sequences.

2.1.5 Vectors

PCR amplified fragments were cloned in the *pCR2.1* TOPO-TA vector, Invitrogen, (Carlsbad, CA). The TOPO-TA vector consists of a linearised vector with a 3' T overhang which is complementary to the 3' A overhang produced by the Taq polymerase. The vector has the DNA topoisomerase I enzyme covalently attached to the phosphate group of each 3' thymidine. When Taq amplified DNA is incubated with the vector the topoisomerase ligates it into the vector which can then be directly used to transform *E. coli*. *pENTR-1A* from Invitrogen (Carlsbad, CA) was used as the base vector for creating the entry cassette vectors described in this thesis. *pB2GW7*, *pK2GW7* and *pH2GW7* (a gift from the Laboratory of Plant Systems Biology; Gent, Belgium) are binary vectors for Agrobacterium-mediated plant transformation. They contain a Gateway destination cassette, a 35S constitutive promoter and resistance cassettes for basta, kanamycin and hygromycin, respectively (Karimi et al., 2002). *pMDC7* (a gift from Ueli Grossniklaus; Zurich, Switzerland) is also a Gateway binary vector, but has an XVE inducible promoter for β-estradiol inducible expression in plants (Zuo et al., 2000). All four binary vectors described here are derived from the *pPZP200* binary vector (Hajdukiewicz et al., 1994).

2.1.6 Bacterial Strains

The *E. coli* strain TOP10 (F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) [*Φ80lacZΔM15*] Δ*lac*X74 *rec*A1 *ara*D139 *gal*U, *gal*K Δ(*ara-leu*)7697 *rps*L (Str^R) *end*A1 *nup*G) (Invitrogen, Carlsbad CA) was used for cloning and plasmid isolation. *E. coli* strain DB3.1 (F⁻ *gyr*A462 *end*A1 Δ(sr1-*rec*A) *mcr*B *mrr hsd*S20(r$_B$-, m$_B$-) *sup*E44 *ara*-14, *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^R) *xyl*-5 λ- *leu mtl*1) was used for plasmid isolation of Gateway vectors containing the *ccdB* toxicity gene. *A. tumefaciens* strain GV3101 harbouring the pMP90 Ti plasmid was used for plant transformation.

2.1.7 Bacterial Growth

E. coli cells were cultured at 37°C in Luria-Bertani (LB) broth (1% (w/v) casein peptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl) with the appropriate antibiotic selection (50 µg ml^{-1} ampicillin, 50 µg ml^{-1} kanamycin or 100 µg ml^{-1} spectinomycin). Cell selection was carried out using LB-agar plates (LB broth + 1.5% (w/v) agar) with the appropriate antibiotic selection. *A. tumefaciens* cells were cultured at 30°C in Yeast-Extract-Peptone (YEP) broth (1% (w/v) meat peptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl) or on YEP-agar plates (YEP broth + 1.5% (w/v) agar) with antibiotic

selection (50 µg ml^{-1} rifampicin, 25 µg ml^{-1} gentamycin and 100 µg ml^{-1} spectinomycin).

2.2 Preparation of Constructs

2.2.1 Cloning

The constructs used in this thesis were prepared using standard methods described in (Sambrook et al., 1989) or, when a commercial kit was used, according to the protocol supplied by the manufacturer. PCR was used to amplify constructs using an existing plasmid or a tomato cDNA library as a template. PCR products were directly ligated into *pCR2.1* Topo-TA cloning vector, the plasmid purified using QIAprep Spin miniprep kit (Qiagen; Hilden, Germany) and the identity of the construct confirmed by sequencing (In-house sequencing service). Constructs were excised from the cloning vector using restriction sites introduced during the PCR amplification and ligated into a *pENTR-1A*-derived vector using complementary restriction sites. Alternatively, very short constructs (such as Strep tag) were made by annealing two single strand oligonucleotides together, and these were designed such that on annealing they represented a sequence identical to that obtained by digestion with the desired restriction enzyme. This allows them to be ligated directly into a vector digested with the same restriction enzymes.

2.2.2 Entry cassettes

pENTR-Stop was created by ligating a 30 bp section of double stranded (annealed) oligonucleotides into the *pENTR-1A* vector between the *Bam*H I and *Xho* I sites. This removes the *ccdB* toxicity gene and introduces a *Not* I site followed by a stop codon. N-terminal tags were PCR amplified with primers that introduce a 5' *Sal* I site and a 3' *Bam*H I site, and these were cloned into the *Sal* I – *Bam*H I sites of *pENTR-Stop*. This allows genes of interest to be cloned into the resulting vector between the *Bam*H I and *Not* I sites without a stop codon, because one is present immediately after the Not I site (Fig 3.1a).

C-terminal tags were PCR amplified with primers that introduce a 5' *Not* I site and stop codon at the 3' end of the tag followed by an *Xho* I site. These were cloned into the *Not* I – *Xho* I site of *pENTR-Stop*. This allows genes of interest to be cloned into the resultant vector between the *Bam*H I and *Not* I sites without a stop codon, because one is present immediately after the tag (Fig 3.1a).

EYFP and *ECFP* tags were cloned from *pEYFP-N1* and *pECFP* (Clontech; Palo Alto, CA). I obtained *GST* from *pGEX-4T-3* (GE healthcare; Little Chalfont, UK), 6x*myc* form *pBIN19-myc-tDET1* (Pierre LaFlamme), 3x*HA* from *pGEX-2T-HA-PtCRY* (Manuela Mangogna), while *STREP* tags were created directly from annealed oligonucleotides. Sequences of assembled entry cassettes were verified by sequencing.

2.2.3 Genes

Genes of interest (see Appendix B) were PCR amplified from a cDNA library or existing constructs using primers that introduce a 5' *Bam*H I site and a 3' *Not* I site. No stop codon was included in the construct unless it was known that the protein is non-functional with a C-terminal tag (e.g., DET1 (Schroeder et al., 2002)) or that the C-terminal amino-acid is known to be important for functional activity of the protein (e.g., ubiquitin). PCR products were ligated into the *pCR2.1* vector using the Topo-TA cloning system and sequenced. Sequenced genes were then excised from the pCR2.1 vector using the *Bam*H I and *Not* I sites introduced during the PCR amplification and ligated into the entry cassette containing the desired tag using the same two restriction sites. The identity of the resulting gene-tag fusions were verified by PCR screening and restriction digests.

2.2.4 Gateway recombination

Tag-gene fusions were transferred from the entry cassette to a plant binary destination vector with the desired promoter and selective marker using the Gateway LR reaction and the Recombinase enzyme (Invitrogen; Carlsbad, CA). The identity of recombined vectors was verified by PCR screening and restriction digests.

2.3 Transgenic lines

2.3.1 *Agrobacterium* transformation

Chemically-competent *Agrobacterium* cells (GV3103 pMP90) were prepared using the CaCl$_2$ method (Weigel and Glazebrook, 2002) and stored at -80°C. 10 ng of binary vector to be transformed was placed in an Eppendorf tube on ice, 50 μl of freshly thawed competent cells were added and the Eppendorf tube transferred to liquid nitrogen. Cells were transferred to a 37°C water bath for 5 min, 1 ml of YEP media added and the cells transferred to a 30°C incubator for two hours. Cells were plated on YEP-Agar plates with appropriate selection (50 μg ml^{-1} rifampicin; 25μg ml^{-1}

gentamycin; 100 µg ml^{-1} spectinomycin) and grown for 48 hours at 30°C. Identity of the resistant clones was confirmed by PCR screening.

2.3.2 Plant Growth

Arabidopsis (ecotype Columbia) plants were grown in soil in a growth room (20°C, 70% humidity) under long day conditions (16/8 hrs light dark cycle).

2.3.3 Plant transformation

Plant transformation was by the floral dip method (Clough and Bent, 1998) and was carried out approximately 12 days after bolting, when the plants have many immature flower clusters, but not many fertilized siliques.

A 250 ml YEP culture was inoculated from an overnight starter culture of *A. tumefaciens* containing the binary vector of interest and grown until OD$_{600\ nm}$ ≥1.0. Cells were pelleted by centrifugation (3 000 *g*, 10 min at room temperature) and resuspended in 100 ml of 5% (w/v) sucrose solution. 0.05% (v/v) Silwet L-77 (Leahe seeds; Round Rock, TX) was added to the solution. *Arabidopsis* inflorescences were then dipped into this solution for 5-10 seconds. After dipping plants were laid on their side, covered in plastic film (to maintain high levels of humidity) and placed in the dark for 24 hrs. This treatment was repeated a second time 10 days later, after which plants were grown to maturity.

2.3.4 Selection of transformants

Seeds (T1) were surface sterilized by washing in 70% (v/v) ethanol containing 0.05% (w/v) SDS for 5 min, 70% (v/v) ethanol for 5 min and then absolute ethanol for 5 min. After sterilization seeds were sown on Petri dishes containing autoclaved Murashige and Skoog (MS) ½ Basal Medium (0.5 x MS salt mixture (Invitrogen; Carlsbad, CA), 1 % (w/v) sucrose, 0.05 % (w/v) MES (pH 5.7), 0.8% (w/v) agargel and 1.12 g ml^{-1} B5 vitamins (Duchefa; Haarlem, Netherlands). For selection of transgenic lines plates were supplemented with 250 µg ml^{-1} carbenicillin and appropriate selective agent (50 µg ml^{-1} kanamycin, 10 µg ml^{-1}, hygromycin B or 10 µg ml^{-1} basta). The Petri dishes were placed in the dark at 4°C for 3 days to break dormancy and then transferred to a growth room at 20°C under long day conditions (16/8 hrs light dark cycle). Seedlings were scored for resistance 10 days after transfer to the growth room. Resistant seedlings were transferred to soil, allowed to self and grown to maturity.

2.3.5 Selection of single locus T-DNA insertions

T2 seed was sown on selective media as described above and segregation ratios were scored 10 days after transfer to the growth room. Segregation data was scored using a single tail chi-squared statistical test to ensure the 3:1 segregation ratio was statistically significant (given significance level (0.05)) and that other segregation ratios did not fit the data (e.g., 1:2, 15:16). Lines that displayed a 3:1 segregation ratio were transferred to soil, allowed to self and grown to maturity (Table 3.1).

2.3.6 Selection of homozygous lines

T3 seed was sown on selective media, and scored for segregation 10 days after transfer to the growth room. Lines that yielded 100% resistant progeny were considered homozygous and used for further experiments.

2.3.7 Screening for expression

2 or 3 rosette leaves (approximately 2 cm^{-2} in total) were collected from individual 3 week old homozygous lines. Leaves were placed in a 1.5 ml Eppendorf tube, liquid nitrogen was added, and the leaves ground to powder with a micro-pestle (VWR; San Diego, California). 50 µl 1 x Laemmli buffer (62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) β-mercaptoethanol, and 0.0005% (w/v) bromophenol blue) was added and the material was ground further. Eppendorf tubes were placed on a heat block at 100°C for 5 min, centrifuged (13 000 g, 2 min, room temp), and the supernatant run on an SDS-PAGE gel. Proteins were transferred to PVDF membranes (45 µM pore size) by dry transfer (2 mA cm^{-2} for 90 min at 4°C). Homogeneous loading and transfer of proteins was verified by staining the membrane with Ponceau-S dye. After washing in PBS to remove Ponceau-S dye, the membrane was blocked (3% (w/v) non-fat skim milk powder in PBS) for 60 min at room temperature. The membrane was incubated with the primary antibody in 1% (w/v) non-fat skim milk powder in PBS) overnight at 4°C. After washing 3 x 5min with PBST (PBS + 0.05% (v/v) Tween 20 detergent) the membrane was incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Pierce; Rockford, IL) at a dilution of 1:10 000 in 3% (w/v) non-fat skim milk powder in PBS for 45 min at room temperature. After washing 3 x 5min with PBST the membrane was incubated in ECL-plus (Enhance chemiluminescence reagent-plus (GE healthcare; Little Chalfont, UK) reagent for 5 min. Images of immunolabelled proteins on the membrane were acquired using a FlourS-Max Imaging system (Bio-rad Laboratories; Hercules, CA) equipped with a cooled CCD camera. Image acquisition was

performed using Quantity-one software (Bio-rad Laboratories; Hercules, CA) and a 15 min exposure time. Images were prepared for presentation using ImageJ 1.37 software (Wayne Rasband, http://rsb.info.nih.gov/ij/).

2.4 Cul4 coimmunoprecipitation experiments

1 g of 3-4 week old myc-tDET1 seedlings were transferred to a mortar containing liquid nitrogen and ground to a fine powder. 2 ml of grinding buffer (50 mM Tris-HCL (pH 8.0), 10 mM $MgCl_2$, 150 mM NaCl, 0.1% (v/v) NP40, 1 mM ß-mercaptoethanol, 0.2 mM AEBSF, Roche complete EDTA-free protease inhibitors) was added and the seedlings ground further. The solution was centrifuged (13 000 g, 10 min at 4°C), the supernatant transferred to a new tube and centrifuged again (13 000 g, 5 min at 4°C). The supernatant was removed and divided into two new tubes. 10 µl of rabbit anti-Cul4 serum was added to one of the tubes (the other served as the no antibody control) which were incubated overnight at 4°C with gentle rotation. 30 µl of protein-A sepharose beads (GE healthcare; Little Chalfont, UK) were added and the tube incubated at 4°C with gentle rotation for 60 min. Beads were washed three times with grinding buffer and the proteins eluted by boiling 10 min in 1x Laemmli buffer and run on a 10% SDS-PAGE gel. Western blotting was preformed as described above in section 2.3.7

2.5 Immunolocalisation

2.5.1 Basic Protocol

Rosette leaves form 10 three week old plants were placed in digestion solution (1% (w/v) cellulase, 0.25% (w/v) macerozyme, 10 mM MES (pH 5.7), 0.4 M mannitol, 30 mM $CaCl_2$, 5 mM β-mercaptoethanol and 0.1% (w/v) BSA) and cut into 3-4 pieces. Vacuum was applied (using a vacuum chamber) for 2 min. The vacuum was released and the leaves incubated for 3 hours in the dark on a rotary shaker (15 rpm) at room temperature. The speed of the shaker was increased to 100 rpm for 10 min to release protoplasts and the solution filtered through a 75 µm mesh filter. The protoplasts were pelleted by gentle centrifugation (2 g, 10 min, room temperature) and the supernatant discarded. The protoplasts were gently resuspended in 10 ml of wash buffer (4 mM MES (pH 5.7), 2 mM KCl and 0.5 M mannitol) and again pelleted by centrifugation (2 g, 10 min, room temperature). After one more wash, a large drop

of protoplast-containing solution was placed on a poly-lysine coated coverslip in a moist chamber and the protoplasts allowed to settle for 1 hour. Protoplasts were fixed by transferring the coverslips to fixation solution consisting of 2% (w/v) paraformaldehyde dissolved in PHEM buffer (6 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl$_2$ (pH 6.9)) for 10 min at room temperature. Coverslips were incubated in 0.5% (v/v) NP40 in PHEM buffer for 5 min at room temperature, then post fixed in ice-cold methanol/acetic acid (50:50) for 10 min at -20°C. Protoplasts were rehydrated by incubating the coverslip in PBS (3 x 5 min) and then transferred to blocking solution (2% (w/v) BSA in PBS) for 30 min at 37°C. Cover slips were incubated with primary antibody (diluted 1:100 in PBS containing 1% (w/v) BSA) for 2 hours at room temperature and then washed once with PBS, once in PBS with 0.1% NP40 and again with PBS (5 min for each wash). Cover slips were incubated with the Alexa 488-coupled goat anti-rabbit antibody (Molecular Probes; Eugene, OR) diluted 1:200 in 0.5% BSA (w/v) in PBS for 45 min at room temperature and then washed once with PBS, once in PBS with 0.1% NP40 and once again with PBS. Coverslips were mounted in Vectorshield H-1000 containing 2 µg ml^{-1} DAPI on microscope slides and sealed with nail varnish.

2.5.2 Soluble protein extraction

Protoplast permeablization prior to fixation can be performed to determine whether a protein is soluble or whether it is retained within a particular cellular fraction, such as the chromatin fraction or cytoskeletal fraction. For this, the immunolocalisation procedure was performed as normal except that after allowing the protoplasts to settle on coverslips for an hour, the coverslips were incubated in 1% (v/v) Triton-X 100 in PBS for 10 min at room temperature. After washing for 1 min in PBS to wash out the soluble proteins, cells were fixed and processed as described in the immunolocalisation method.

2.5.3 Image acquisition

Protoplasts were imaged on an inverted microscope (Axiovert 135TV, Zeiss; Jena, Germany) with a 1.3 NA 100x lens, and DAPI (Zeiss 001) and FITC filter sets (Zeiss 009). Images were collected with a 12-bit, grey-scale, cooled-CCD camera (ORCA-100, Hamamatsu; Hamamatsu City, Japan) controlled by Openlab 4 software from Improvision (Coventry, UK). For each channel a z-stack of approximately 60 focal planes was acquired with a z-step of 200 nm and 1x camera binning, resulting in x and y pixel dimensions of 67 nm. Image stacks were deconvolved using the maximum-likelihood estimation-maximum algorithm (Conchello and McNally, 1996)

as implemented in the ImageTrak 2.2 program (Peter K. Stys, http://www.ohri.ca/stys/imagetrak). This method was run for 250 iterations using a theoretical point-spread-function (PSF, a model of the blur introduced during the image acquisition). Similar results were obtained using other deconvolution algorithms such as Nearest-Neighbours deconvolution or Weiner filtering. Stacks were false-coloured, merged and prepared for presentation using ImageJ 1.37 (Wayne Rasband, http://rsb.info.nih.gov/ij/).

2.6 Chromatin Immunoprecipitation

2.6.1 Basic Protocol

Chromatin immunoprecipitation (ChIP) was adapted from (Bowler et al., 2004). All procedures were performed on ice or at 4°C unless otherwise stated. 1.5 g of wild type or myc-tDET1 seedlings were submerged in 37 ml of fixation solution at room temperature (1% (v/v) formaldehyde, 0.4 M sucrose, 10 mM HEPES, (pH 8.0)) in a 50 ml Falcon tube and vacuum was applied for 10 min (for dark grown seedlings this step was performed under dim green safe-light conditions). The cross-linking was stopped by adding 2.5 ml of 2 M glycine and application of vacuum for another 5 min. Seedlings were rinsed twice in 40 ml of ice-cold DDW, dried on tissue paper, and frozen in liquid nitrogen. Seedlings were then transferred to a pre-cooled mortar, ground to a fine powder in liquid nitrogen and transferred to a 50 ml Falcon tube containing 37 ml of ice-cold extraction buffer 1 (0.4 M sucrose, 10 mM HEPES (pH 8.0), 5 mM ß-mercaptoethanol, 0.2 mM AEBSF and Roche complete EDTA-free protease inhibitors). The extract was filtered through 2 layers of miracloth (VWR; San Diego, California) and centrifuged (3 000 g, 20 min, 4°C). The supernatant was discarded and the pellet resuspended in 1 ml of ice-cold extraction buffer 2 (0.25 M sucrose, 10 mM HEPES (pH 8.0), 10 mM MgCl2, 1% (v/v) Triton X-100, 5 mM ß-mercaptoethanol, 0.2 mM AEBSF, Roche complete EDTA-free protease inhibitors), transferred to a 1.5 ml Eppendorf tube and centrifuged (12 000 g, 10 min, 4°C). The pellet was resuspended in 300 µl of ice-cold extraction buffer 3 (1.7 M sucrose, 10 mM HEPES (pH 8.0), 0.15% (v/v) Triton X-100, 2 mM MgCl2, 5 mM ß-mercaptoethanol, 0.2 mM AEBSF, Roche complete EDTA-free protease inhibitors), overlaid onto another 300 µl of extraction buffer 3 in a 1.5 ml Eppendorf tube and centrifuged (16 000 g, 60 min, 4°C). The supernatant was discarded and the pellet resuspended in 300 µl of room-temperature nuclei lysis buffer (50 mM HEPES (pH

8.0), 10 mM EDTA, 1% (w/v) SDS, 0.2 mM AEBSF, Roche complete EDTA-free protease inhibitors) and incubated on ice for 20 min. 2.7 ml of ChIP Dilution Buffer (1.1% (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM HEPES (pH 8.0), 167 mM NaCl, 0.2 mM AEBSF, Roche complete EDTA-free protease inhibitors) was added and the solution sonicated to shear DNA to approximately 0.5-2 kb DNA fragments. Sonication was performed 7x10 seconds (30% output on a Branson Sonifier 250 (Branson Ultrasonics; Danbury, CT) fitted amplification horn and a 5mm microtip) in an ethanol/ice bath with a 50 second pause between bursts. The sonicated chromatin was centrifuged (16,000 g, 5 min 4°C) and the supernatant transferred to a new tube. The chromatin solution was pre-cleared by adding 40 μl of salmon sperm sheared DNA/Protein A Agarose beads, Upstate (Lake Placid, NY) and incubating for 1 hour at 4°C on a rotating wheel. The chromatin/beads solution was centrifuged (16,000 g, 5 min 4°C) and the supernatant split into 3 tubes. 1-2 μg of antibody was added to two of the tubes while the 3rd was used as the no-antibody control. The tubes were incubated overnight at 4°C on a rotating wheel. 40 μl of salmon sperm sheared DNA/Protein A Agarose beads was added and the tubes rotated for another hour at 4°C. The beads were pelleted by centrifugation (27 g, 2 min, 4°C), washed twice with low salt buffer (150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM HEPES (pH 8.0)), twice with high salt buffer (500 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM HEPES (pH 8.0)), twice with LiCl buffer (0.25 M LiCl, 1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 1 mM EDTA, 10 mM HEPES (pH 8.0)) and twice with TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). After the final wash 250 μl of elution buffer (1% (w/v) SDS, 100 mM NaHCO$_3$) was added to the beads and the resultant slurry was incubated at 65°C for 15 min with gentle agitation. After a brief centrifugation to pellet the beads, the supernatant was transferred to a new tube, the extraction was repeated by adding another 250 μl of elution buffer to the beads. The two elutions were then combined. 20 μl of 5M NaCl was added to the eluant, which was then incubated overnight at 65°C to reverse the crosslinking. In the morning 20 μl of proteinase K buffer (250 mM EDTA, 1 M Tris-HCl (pH 6.5)) and 2 μl of proteinase K (10 mg ml^{-1}) were added and the solution incubated at 45°C for 3 hours. After phenol extraction the DNA was ethanol precipitated and resuspended in 50 μl of DDW plus RNAaseA.

2.6.2 Semi-quantitative PCR of ChIP samples

Enrichment of loci of interest in the immunoprecipitated chromatin was detected by semi-quantitative PCR. Oligonucleotide primers that amplify the *Ta2* retrotransposon

(5'-AAACGATGCGTTGGGATAGGTC-3' and 5'-ATACTCTCCACTTCCCGTTT TTCTTTTTA-3') and Actin2/7 gene (5'-CGTTTCGCTTTCCTTAGTGTTAGCT-3' and 5'-AGCGAACGGATCTAGAGACTCACCTTG-3') were as previously described (Johnson et al., 2002). Primers that amplify the *CAB2* promoter (5'-AAAACTGGTTCGATAGTGTTG-3' and 5'-CATTCT TGTCACGAGGGTGT-3') were as previously described (Bertrand 03). Primers that amplify the *HEMA1* promoter (5'-ACCAAACCTTTGCGAGAGAG and 5'-TGCCGTGTAAGAACAAATGC-3'), the *POR-A* promoter (5'-GCTTCGATGAAAGTCTGTGCT-3' and 5'-TCATGGGACTCCATCTC TTTG-3') and the *FT* promoter (5'-TTGGCGGTACCCTACTTTTT-3' and 5'-CGGGTCGGTGAAATCATAAC-3') were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3www.cg). Standard settings, except for setting minimum and maximum primer GC content at 40% and 60%, respectively, and setting amplified fragment length to 250-350 base-pairs were used. 1.5 µl of sample DNA from each chromatin immunoprecipitation was used in each 20 µl PCR reaction of 20 µl, (1 unit of Taq DNA polymerase (Biogem; Naples, Italy), 1.5 mM MgC$_2$, 1 pM of each oligonucleotide primer, 1 nM dNTP's). PCR was performed using a programmable thermocycler (PTC-100, MJ Research; Waltham, MA) using the following scheme : 94°C for 3 min once, followed by cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min for X times, and then once at 72°C for 5 min. This number of PCR cycles (31) was determined empirically as the number that did not give any signal in the no antibody control but gave a robust signal from total genomic DNA equal to 0.1% of that used in each chromatin immunoprecipitation. For each locus the PCR assay was performed in triplicate and each chromatin immunoprecipitation was performed at least twice.

3. Results

3.1 Gateway Cloning System

3.1.1 Introduction

As part of a wider investigation into the function of the DET1 complex and chromatin remodelling in plants it was of interest to generate transgenic Arabidopsis lines over-expressing a number of different proteins from tomato (DET1, DDB1, DDB2, H2A, H2B, and Ubiquitin) with a number of different tags: EYFP, ECFP, (for interaction/localisation studies), and 6xMYC, GST, Strep and 3xHA (for co-immunoprecipitation, chromatin immunoprecipitation and complex purification experiments). Cloning each of the required fusions on a one-by-one basis would be prohibitively time consuming, so I designed and created a medium-throughput cloning system based on Gateway DNA-recombination technology.

3.1.2 Considerations

Agrobacterium-mediated plant transformation requires the use of binary vectors containing the construct of interest, promoter, terminator, and plant selectable marker within the borders of the transfer-DNA, bacterial selectable marker, *A. tumefaciens* origin of replication and *E. coli* origin of replication. Because of these requirements the resulting binary vectors are large (typically 10-19 kb), difficult to manipulate and contain few restriction sites that can be useful for cloning (Karimi et al., 2002), making manipulation of these vectors an awkward and time-consuming process. In addition, because it is now well established that adding a tag to either the amino or carboxy terminus of a protein can adversely affect it's activity in a manner that is difficult to predict, it is desirable to try both an amino and carboxy tag for each protein-tag fusion in case one of the fusions interferes with the folding or the function of the protein.

In order to overcome the difficulties of cloning into plant binary vectors some investigators have shifted to using the Gateway cloning system commercialised by Invitrogen (Carlsbad, CA). In this system the construct of interest is first cloned into a minimal ENTRY vector (pENTR) and is then transferred to a binary DESTINATION vector (pDEST) by site-specific recombination. This is done using the Int and Xis recombination proteins that recognise the *attL* and *attR* sites in entry and destination vectors, respectively, and that recombine them to generate an *attB* site. The *att*

sequences contain two parts that are separated by an intervening nucleotide sequence, and it is in this region that the multiple cloning site (MCS) is located in the entry vector (Fig 3.1a). The result of this is that any gene cloned into the MCS is transferred from the ENTRY to the epitope tag containing DESTINATION vector during the course of the recombination (Hartley et al., 2000). While this strategy is increasingly used for creating vectors for plant transformation (Karimi et al., 2002) (Curtis and Grossniklaus, 2003) (Earley et al., 2006), it is not without its drawbacks, some of which include a limited number of epitope tags available, the presence of a bulky and often highly-charged linker sequence between the tag and the protein and, in the case of N-terminal fusions, between the protein and stop codon. This is problematic in the case of proteins such as DET1, where it has been demonstrated previously that C-terminal tag-protein fusions are not functional (Schroeder et al., 2002).

In order to overcome these limitations we decided to develop a hybrid gateway cloning system were the tag is already present in the entry vector either 5' or 3' of the MCS with a stop codon in the appropriate position (Fig 3.1a). This results in the entire open reading frame contained within the ENTRY vector. Using Gateway recombination the ORF containing the protein-tag fusion is then transferred to the DESTINATION binary vector between the promoter and terminator (Fig 3.1b) (Parr and Ball, 2003). The major advantage of this strategy is that, unlike conventional Gateway cloned constructs, the ORF does not include the *att* sequences and the long and highly charged polylinker sequences that they give rise to. While these constructs were designed primarily for plant transformation, they can also be recombined into expression vectors for any other organism of choice (e.g. mammalian cells, insect cells or bacteria (Hartley et al., 2000)). A final consideration is that these constructs will be used for *Agrobacterium*-mediated transformation of Arabidopsis plants. In order to reduce the risk of trans-silencing between the transgene and the endogene e.g., (Davuluri et al., 2004) we decided to clone the genes of interest from tomato which should be sufficiently different from their Arabidopsis homologs at the nucleotide level to avoid such trans-silencing effects.
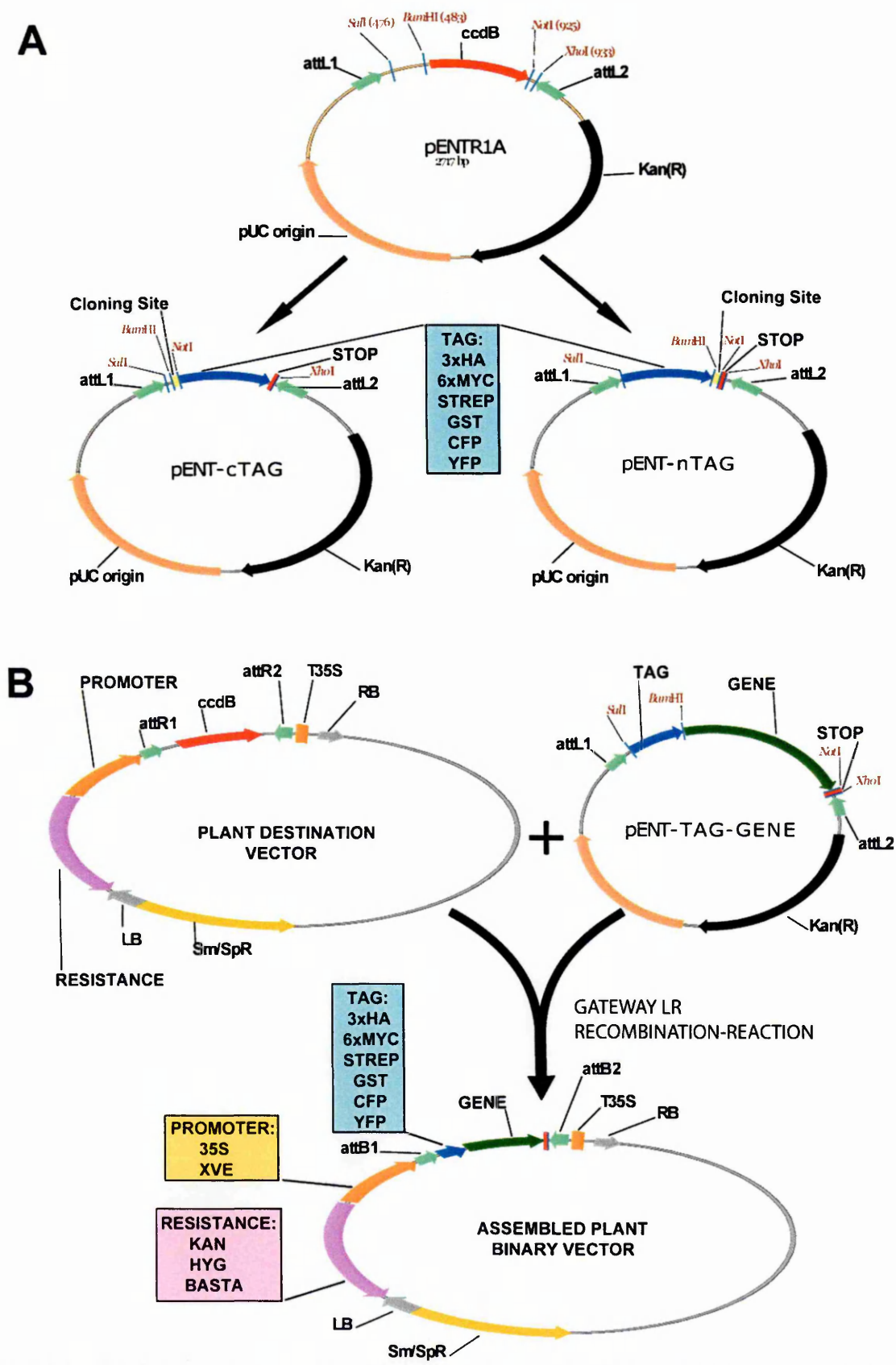
Figure 3.1 (A) Schematic showing the design of the modified Gateway ENTRY cassettes derived from the *pENTR-1A* vector (Invitrogen). 6 different tags were each cloned in the 5' and 3' position, resulting in 12 different ENTRY cassettes. **(B)** Schematic showing an entry cassette containing a gene of interest being recombined with a Gateway compatible plant DESTINATION vector. Depending on ENTRY cassette and DESTINATION vector used different combinations of selectable marker, promoter and tag are possible.

3.1.3 Design

Entry vectors

Modified Gateway entry cassettes were designed by introducing sequences encoding each of the epitope tags into the pENTR-1A vector (Invitrogen) in place of the *ccdB* toxicity gene so that they contained the tag either 5' or 3' to the multiple cloning site (MCS) with a stop-codon immediately after the MCS (for 5' tags) or after the tag for 3' tags (Fig 3.1a). This results in the complete Open Reading Frame contained within the entry vector. The 6 tags cloned were Enhanced yellow fluorescent protein (EYFP), Enhanced Cyan Fluorescent Protein (ECFP) (for interaction/localisation studies), 6xMYC (the C-terminal epitope of the Myc onco-protein) and 3xHA (*Influenza hemagglutinin*) epitope tags, and STREP (streptavidin) and *Schistosoma japonicum* glutathione *S*-transferase (GST) affinity tags. Each tag was cloned in both the 3' and 5' configuration resulting in 12 different entry cassettes. The vectors were designed so that when the protein of interest is cloned into the MCS using the *Bam*H I and *Not* I restriction sites a Gly-Gly-Ser-Gly-Gly poly-linker is present except for ECFP and EYFP (no polylinker present) and the 3' GST tag (which has a thrombin cleavage site Leu-Val-Pro-Arg-Gly-Ser in place of the polylinker).

Destination vectors

The design of the entry cassettes is such that when the protein of interest is cloned into the MCS the entire open reading frame is contained within the entry cassette which can then be recombined with any Gateway destination vector containing a suitable promoter and terminator. In the context of this study the destination vector was usually a plant binary vector with suitable promoter and terminator sequences, but the system is also compatible with common overexpression vectors for bacteria (*pDEST14*), mammalian (*pDEST32*) and insect cell overexpression. Binary vectors derived from *pPZP-200* (Hajdukiewicz et al., 1994) with a Gateway recombination cassette were obtained from The Laboratory of Plant Systems Biology (Gent, Belgium) (Karimi et al., 2002). These vectors have a 35S Cauliflower Mosaic Virus (CaMV) promoter/terminator (Odell et al., 1985) and either a hygromycin-B resistance cassette (*pH2GW7*), a kanamycin resistance cassette (*pK2GW7*) or a Basta resistance cassette (*pB2GW7*). A 4th vector, *pMDC7*, was obtained from Ueli Grossniklaus (Zurich, Switzerland) which has a 17-β-estradiol inducible promoter (XVE; (Zuo et al., 2000) and a hygromycin resistance cassette (Curtis and Grossniklaus, 2003). The XVE promoter consists of a fusion protein consisting of the DNA binding domain of the LexA protein (X), the VP16 acidic transactivating domain

and the regulatory region of the human estrogen receptor (E). The constitutive G10-90 promoter is used to express this chimeric fusion protein, which under normal conditions is targeted to the plasma membrane by the regulatory region of the human estrogen receptor. Uptake of 17-β-estradiol (an estrogen analogue) inactivates the regulatory region allowing translocation to the nucleus where the LexA DNA binding domain targets the fusion to a LexA operator fused upstream of a minimal (-46) 35S promoter (which is in turn upstream of the *attR* recombination site). The VP16 domain then activates the minimal 35S promoter and drives expression of the transgene (Zuo et al., 2000).

3.1.4 Generation of Constructs for Transformation

The following genes were amplified from existing constructs or a tomato (*Solanum lycopersicon*) cDNA library: *H2A, H2B, Ubiquitin, DET1, DDB1* and *DDB2*. The primers used were designed so that a *Bam*H I restriction site was present immediately upstream of the ATG initiation codon and a *Not* I site introduced in place of the stop codon (except for *DET1* and *Ubiquitin* where it was thought that a C-terminal tag may interfere with protein function (Schroeder et al., 2002) or conjugation, respectively. In these cases the *Not* I site was incorporated immediately 3' of the stop codon. PCR products were cloned into Topo-TA vector and sequenced.

Vector	Promoter	Construct	Resistance	Lines	Express	Homozygous
pHhUBI	35S	ha-tUBI	Hyg	4	yes (4/4)	1
pKgUBI	35S	gst-tUBI	Kan	1	not tested	0
pHcUBI	35S	cfp-tUBI	Hyg	4	yes (2/2)	0
pByUBI	35S	yfp-tUBI	Bas	1	yes (1/1)	0
pBsUBI	35S	strep-tUBI	Bas	5	yes (1/1)	0
pHhH2A	35S	ha-tH2A	Hyg	26	yes (13/13)	2
pH-H2Ah	35S	tH2A-ha	Hyg	15	yes (4/5)	1
pHhH2B	35S	ha-tH2B	Hyg	23	yes (14/14)	0
pH-H2Bh	35S	tH2B-ha	Hyg	6	yes (6/6)	0
pB-H2Bs	35S	tH2B-strep	Bas	4	yes (1/1)	1
pMyDET1	XVE	yfp-tDET1	Hyg	10	yes (5/7)	1
pByDET1	35S	yfp-tDET1	Bas	7	nil (7/7)	1
pBsDET1	35S	strep-tDET1	Bas	5	nil (5/5)	0
pByDDB2	35S	yfp-tDDB2	Bas	3	nil (3/3)	0
pB-DDB2y	35S	tDDB2-yfp	Bas	2	nil (2/2)	0

Table 3.1 Transgenic Arabidopsis lines obtained using Gateway vector system. Lines indicates the number of independent primary transformants obtained. Express, if expression of the transgene was detected with the number of lines for which expression was detected followed by the number of lines tested in brackets. Homozygous indicates the number of independent lines for which plants homozygous for the transgene were identified by segregation analysis.

Once the sequence had been verified the cDNA's were excised from the vector using the *BamH* I and *Not* I restriction sites and cloned into the MCS of the entry cassettes using the same two restriction sites. In this way the following entry cassettes were constructed: *STREP-DET1, EYFP-DET1, EYFP-UBI, ECFP-UBI, GST-UBI, STREP-UBI, 3xHA-UBI, 6xMYC-UBI, 3xHA-H2A, H2A-3xHA, 3xHA-H2B, H2B-3xHA, H2B-STREP, STREP-DDB2, EYFP-DDB2, DDB2-EYFP ECFP-DDB1* and *GST-DDB1*. After the identity of the constructs had been confirmed by restriction analysis they were recombined into the Gateway binary destination vectors to produce expression clones which were verified by PCR colony screening and restriction analysis (Table 3.1).

3.1.5 Generation of Transgenic Arabidopsis Lines

The expression clones were introduced into *Agrobacterium tumefaciens* strain GV3101 using the chemical transformation method (Glazebrook and Weigel). Incorporation of the binary vector into the cells was confirmed by PCR colony screening (Fig 3.2 and data not shown). These *A. tumefaciens* strains were then used to transform *A. thaliana* (ecotype Columbia) using the floral-dip method (Clough and Bent, 1998). Plants were grown to maturity and (T1) seed collected. Seed from transformed plants was sown on MS1/2 plates with the appropriate antibiotic/herbicide selection and independent primary transformants (T1) were obtained as outlined in Table 3.1.
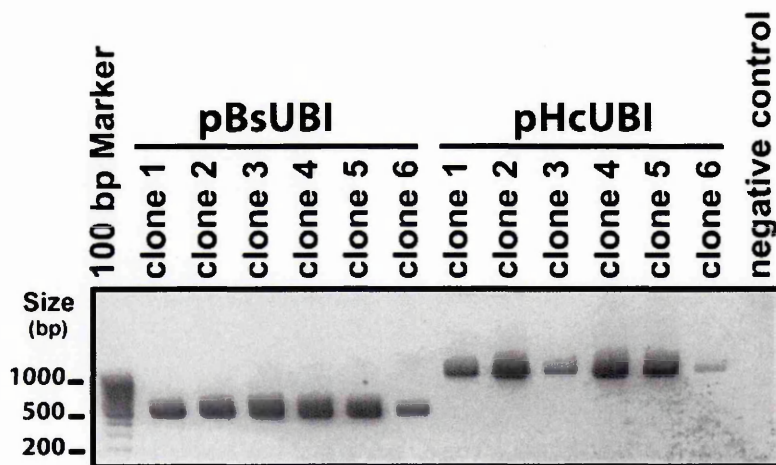
Figure 3.2 PCR colony screen on *Agrobacterium tumefaciens* strain GV3101 chemicaly transformed with pBsUBI or pHcUBI vectors. In the case of positive clones the 35sF and RubiST primers (appendix A) used give rise to a PCR product of 550 bp for pBsUBI or 1050 bp for pHcUBI.
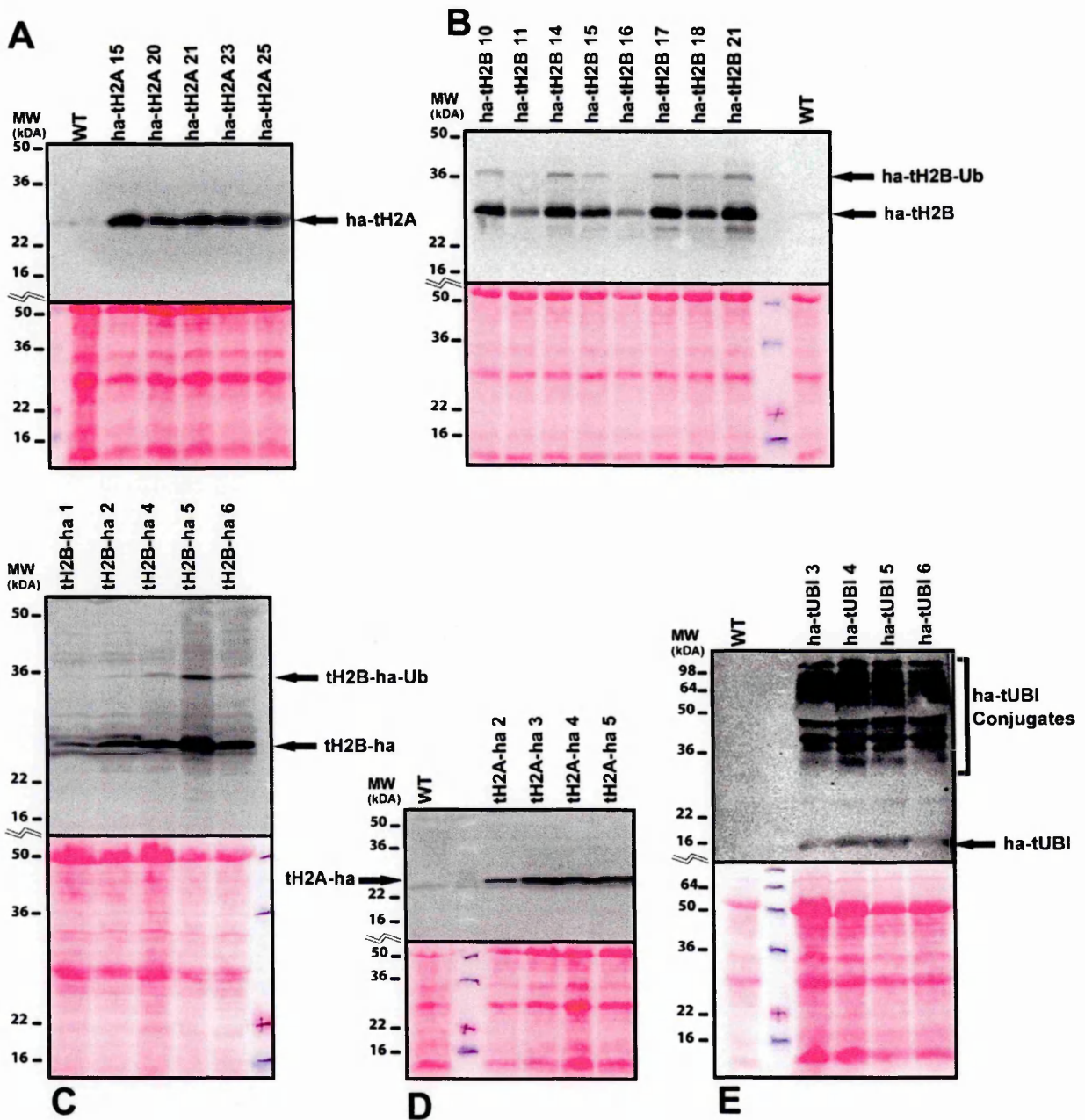
Figure 3.3 Western Blot of SDS-soluble proteins from individual T1 transformed lines. The upper panels show membranes probed with anti-HA antibodies, while the lower panels show the same membranes stained with Ponceau-S solution. **(A)** ha-tH2A transformants. **(B)** ha-tH2B transformants **(C)** tH2B-ha transformants Note that in panels B and C a minor band 8-9 kDa higher than the major band is also present, presumably corresponding to mono-ubiquitinated H2B, a common post-translational histone modification in eukaryotes. **(D)** tH2A-ha transformants. **(E)** ha-tUBI transformants, note minor band at approximately 13 kDa corresponding to free HA-tagged ubiquitin while the majority of ha-tUBI forms higher MW species, presumably due to conjugation to other proteins.

Lines were screened for transgene expression by western-blotting (Fig 3.3, Fig 3.4b) or epi-fluorescence microscopy (Fig 3.4a). Expressing lines were identified for all constructs except the following: GST-UBI (not tested), STREP-DET1, EYFP-DDB2, DDB2-EYFP and EYFP-DET1 (35S promoter). It is noteworthy that for DET1 (which is normally a constiutively expressed protein) we failed to detect expression of the EYFP-DET1 transgene from a 35S promoter construct, but were able to obtain expression of this fusion when it was expressed from the XVE inducible promoter (Fig 3.4). Expression of EYFP-DET1 from this promoter was detected 24 hours after transfer to inductive media and levels of the protein declined over the next 4-5 days to undetectable levels. The difference in expression of the same transgene by the two different promoters may be due to silencing of the 35S driven transgene (Davuluri et al., 2004).
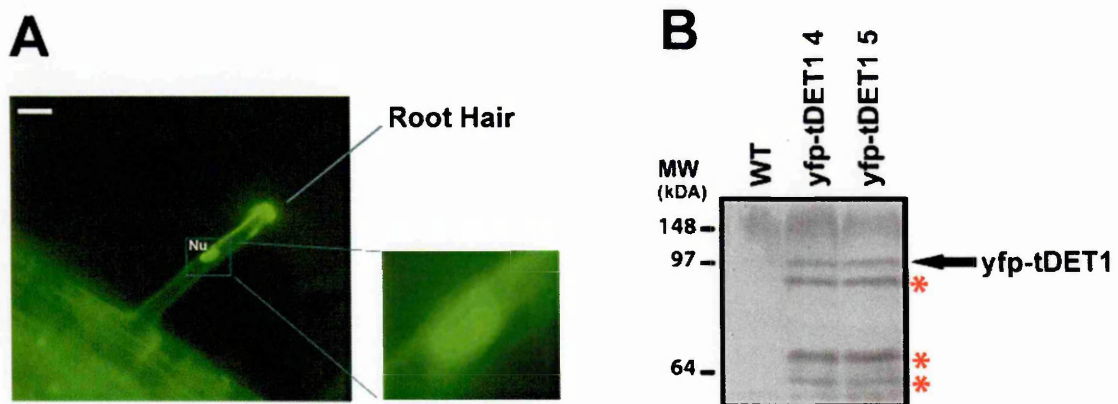
Figure 3.4 (A) Epifluorescence picture showing YFP-tDET1 localized to the nucleus of a root-hair from a XVE::*EYFP-tDET1* line induced with 10 μM β-estradiol, Nu = nucleus, scale bar is 10 μM. **(B)** Western blot of SDS soluble proteins from XVE::*EYFP-tDET1* line induced with 10 μM β-estradiol using an anti-GFP antibody. As well as the full length protein, several degradation products are present (* indicates a degradation product).

On the other hand, the sudden induction of the inducible promoter (by transfer of seedlings to media containing β-estradiol) may initially allow expression of the transgene before silencing can take effect. However the expression of mRNA for the induced transgene eventually triggers silencing as evidenced by the loss of fluorescence over the next 4-5 days. Alternatively the XVE promoter has been reported to express proteins at a level up to 8 times higher than the 35S promoter (Zuo et al., 2000) so this may be responsible for the differing results between the two promoters. In agreement with this, MYC tagged DET1 expressed from a 35S promoter was detected, suggesting that the level of expression of DET1 fusions from this promoter are too low to allow direct detection by protein fluorescence but are sufficient for detection by more sensitive antibody based methods such as Western blot and immunofluorescence (Fig 3.5 and Fig 3.7 respectively). *Arabidopsis* lines positive by microscopy were also analysed by western blot to confirm that the expressed protein was of the anticipated molecular weight (Fig 3.4b). Positive lines were allowed to self-pollinate and T2 seed subjected to segregation analysis (using antibiotic/herbicide resistance) to confirm single locus insertion (data not shown). T3 seed was collected form individual T2 plants and again subjected to segregation analysis to identify pools of homozygous (T3) seed for each line (data not shown). None of these homozygous lines showed any obvious phenotype.

3.2 Characterisation of Transgenic Myc-tDET1 Expressing Plants

3.2.1 Introduction

A central aim of this thesis was to characterise the targets of the DET1 complex at a cellular and molecular level, a task that generally requires an antibody capable of detecting the protein of interest. As attempts to raise an antibody reactive against tomato DET1 have not been successful to date (data not shown), it was decided to employ an epitope-labelling approach. In this method a transgenic line is created in which the protein of interest is fused to a short peptide sequence (epitope) which can be recognised by a commercially available antibody.

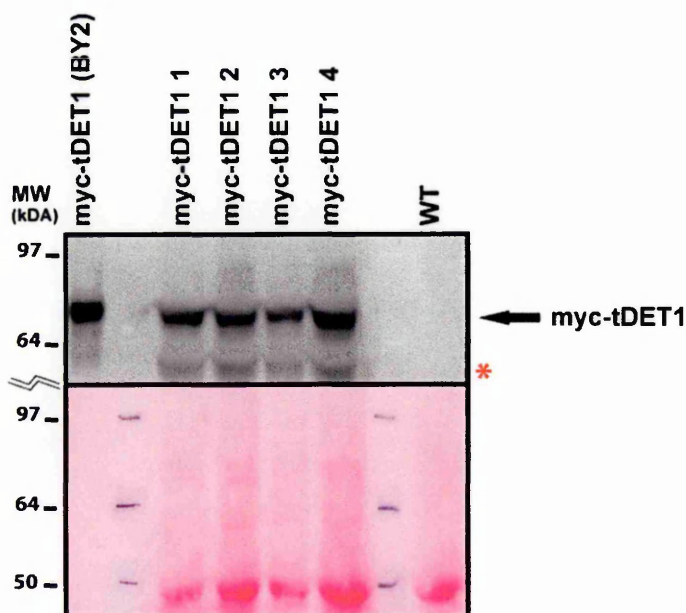**Figure 3.5 (A)** Schematic showing the pBIN19-*35S::myc-tDET1* binary vector (P. Laflamme) used to generate the transgenic myc-tDET1 lines used in this study. **(B)** Western Blot of SDS-soluble proteins from individual T2 myc-tDET1 lines. The upper panel shows the membrane probed with an anti-myc antibody, while the lower panel shows the same membrane stained with Ponceau-S solution. * Indicates a degradation product.

In this case it was decided to use 6 copies of the c-MYC epitope (6xMYC) fused to the N-terminus of DET1 (Fig 3.5a). This construct had been created previously in the lab, inserted into the pBIN-19 plasmid under the control of the 35S (cauliflower mosaic virus) promoter, and used to transform Arabidopsis (ecotype Columbia).

3.2.2 Selection of expressing lines

T1 seed from the primary transformants was sown on MS1/2 media supplemented with Kanamycin and resistant plants transferred to soil and individually propagated. A leaf was taken from each individual line and subjected to western blot analysis with an anti-MYC antibody. Although I was not initially able to detect the protein in extracts from these plants using a commercial anti-myc 9E10 antibody (data not shown), after switching to higher-affinity anti-myc antibodies I was able to confirm by western blotting that the transgene was expressed and of the expected molecular weight (Fig 3.5b). Expressing lines were allowed to self and the T2 seed collected and sown on MS1/2 media supplemented with kanamycin. Segregation analysis (as determined by resistance to kanamycin) on a number of individual lines revealed a 3:1 segregation ratio, indicating single-locus insertions. Single tailed Chi-Squared statistical tests were performed, and confirmed that the attributed segregation ratios were statistically feasible and used to eliminate alternative segregation ratios such as 1:15 or 1:1 (Table 3.2). Plants from these lines were transferred to soil and allowed to self-fertilise. T3 seed was collected, sown and again subjected to segregation analysis (Table 3.3) to select lines where all progeny were kanamycin resistant (indicating that these lines were homozygous).

T2 line	Observed		Predicted						p-values		
	KanR	KanS	3:1 R	3:1 s	1:1 R	1:1 s	15:1 R	15:1 s	3to1	1to1	15to1
mDET1 1	82	31	84.8	28.3	57	56.5	106	7.063	0.55	2E-06	1E-20
mDET1 2	72	30	76.5	25.5	51	51	95.6	6.375	0.303	3E-05	4E-22
mDET1 3	96	27	92.3	30.8	62	61.5	115	7.688	0.435	5E-10	6E-13
mDET1 4	93	25	88.5	29.5	59	59	111	7.375	0.339	4E-10	2E-11

Table 3.2 Segregation analyses on T2 seed from 4 independent lines transformed with the *35S::myc-tDET1* construct indicates they are all single-locus insertions. The number of kanamycin resistant (KanR) and kanamycin sensitive (KanS) seedlings obtained are in the Observed column. Predicted number of resistant (R) and sensitive (S) seedlings for a 3:1, 1:1 and 15:1 segregation ratio are in the Predicted column. For each segregation ratio the predicted values were compared with the observed values using a single-tailed Chi-Squared statistical test and the probability that the observed data was consistent with the segregation ratio calculated (p-value). A p-value above 0.05 is considered statistically significant and is highlighted in green, non-statistically significant P values are highlighted in orange.

T3 line	Observed		Predicted						p-values		
	KanR	KanS	3:1 R	3:1 s	1:1 R	1:1 s	15:1 R	15:1 s	3to1	1to1	15to1
mDET1 1.1	68	29	72.8	24.3	49	48.5	90.9	6.063	0.265	7E-05	6E-22
mDET1 1.2	108	0	81	27	54	54	101	6.75	2E-09	3E-25	0.007
mDET1 1.3	72	34	79.5	26.5	53	53	99.4	6.625	0.093	2E-04	5E-28
mDET1 1.4	89	18	80.3	26.8	54	53.5	100	6.688	0.051	7E-12	6E-06
mDET1 1.5	122	0	91.5	30.5	61	61	114	7.625	2E-10	2E-28	0.004
mDET1 1.6	63	18	60.8	20.3	41	40.5	75.9	5.063	0.564	6E-07	3E-09

Table 3.3 Segregation analysis on T3 seed from 6 self-fertilised T2 plants confirms that lines mDET1 1.2 and mDET1 1.5 are homozygous for the transgene. The number of kanamycin Resistant (KanR) and kanamycin sensitive (KanS) seedlings obtained are in the Observed column. Predicted number of resistant (R) and sensitive (S) seedlings for a 3:1, 1:1 and 15:1 segregation ratio are in the Predicted column. For each segregation ratio the predicted values were compared with the observed values using a single-tailed Chi-Squared statistical test and the probability that the observed data was consistent with the segregation ratio calculated (p-value). A p-value above 0.05 is considered statistically significant and is highlighted in green, non-statistically significant P values are highlighted in orange.

Plants from these homozygous lines were transferred to soil and grown in bulk in order to provide sufficient seed for future experiments. These lines showed no obvious phenotype and were also used for the experiments described in (Bernhardt et al., 2006).

3.2.3 Analysis of myc-tDET1 expressing lines

The next step was to determine whether the myc-tDET1 fusion protein behaved in a manner analogous to the endogenous DET1 protein. Western blots on independent lines showed a band at about 70 kDa (Fig 3.5b), consistent with the predicted molecular weight of DET1 (62 kDa) plus the 6xmyc epitope tag (10 kDa). The myc-tDET1 expressing line was crossed into a *det1* null mutant and resulted in a partial restoration of the wild-type phenotype, thus suggesting that the myc-tDET1 fusion protein is at least partially functional (data not shown). It has been demonstrated previously that in planta DET1 forms part of a complex with DDB1 and COP10 ((Schroeder et al., 2002), (Yanagawa et al., 2004). Data from mammalian-cell experiments show that an analogous complex consisting of human DET1, human DDB1 and human COP1 forms the substrate adaptor for a CUL4-based ubiquitin-ligase complex (Wertz et al., 2004). A CUL4 homolog is present in Arabidopsis (Risseeuw et al., 2003) and so the existence of an interaction between CUL4 and a DET1 containing complex has been widely predicted (Thomann et al., 2005) (Schwechheimer and Villalobos, 2004). In order to assess this possibility, yeast-2-hybrid analysis (preformed by Anne Bernhardt, Free University of Berlin) confirmed that *Arabidopsis* CUL4 and DDB1a (one of two DDB1 homologs in *Arabidopsis*) interact directly with each other (Fig 3.6a).
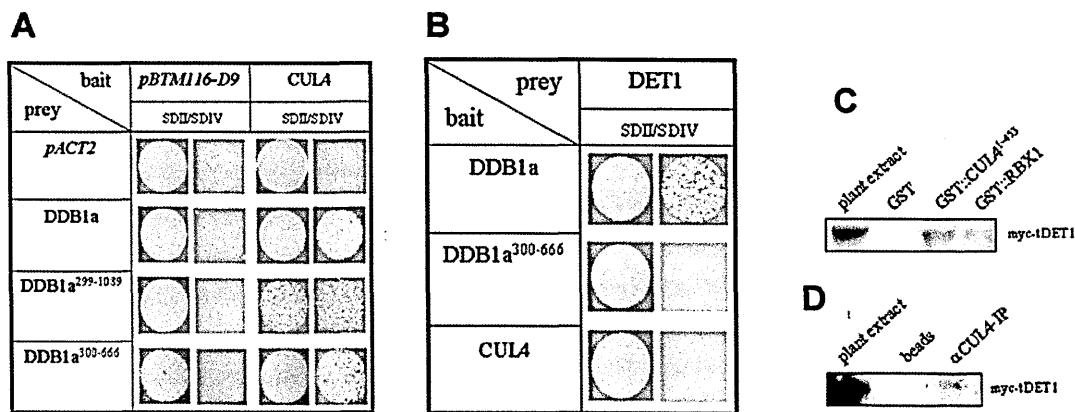
Figure 3.6 (A) Yeast-2-Hybrid analysis of interaction of DDB1 with CUL4). **(B)** Yeast-2-Hybrid analysis of interaction of DDB1 and CUL4 with DET1. SDII, selection medium for transformation with bait (pBTM116-D9) and prey (pACT2) plasmids supplemented with leucine and histidine; SDIV, selection medium for interaction studies without leucine and histidine supplements. Photos were taken from single spots. **(C)** GST-pulldown of myc-tDET1 from plant extracts by GST-CUL4 and GST-RBX1 but not GST alone. **(D)** Co-immunoprecipitation of myc-tDET1 when endogenous CUL4 is immunoprecipitated from plant extracts.

Using deletion constructs of DDB1a it was demonstrated that residues 300-666 of DDB1a are responsible for mediating the interaction with CUL4 (Fig3.5a). Further yeast-2-hybrid assays confirmed that DDB1a and DET1 interacted, as had previously been demonstrated (Schroeder et al., 2002), although DET1 and CUL4 failed to interact (Fig3.6b), indicating that any association between DET1 and CUL4 is likely to be mediated by DDB1a acting as a bridge between the two. These interactions were confirmed *in vitro* using GST pull-down experiments (Bernhardt et al., 2006). Further pulldown experiments using GST-CUL4 and extracts from myc-tDET1 expressing plants confirmed that GST-CUL4 could pull down myc-tDET1 from plant extracts (Fig 3.6c). Finally, in order to confirm the interaction *in planta* we prepared whole cell extracts from myc-tDET1 expressing plants and performed co-immunoprecipitation with serum specific for CUL4 (a kind gift from Pascal Genschik). The immunoprecipitated proteins were probed by western blotting with an antibody against myc (which recognises the myc-tDET1 fusion protein) (Fig 3.6d). A band of 70 kDa was detected in the CUL4 IP lane, but not in the no-antibody control. This indicated that myc-tDET1 was specifically co-immunoprecipitated with CUL4.

These data confirm the existence of a CUL4-based ubiquitin E3 ligase in plants and suggest that DET1 and CUL4 are in the same complex but do not directly interact,

instead the binding partner of DET1; DDB1a bridges the two proteins by via a domain contained within residues 300-666 which binds to CUL4 and an undefined domain which binds DET1. Based on these data we can model the structure of the plant CUL4-based ubiquitin ligase (Fig 4.1). These data also indicate that the overexpressed myc-tDET1 fusion protein appears to be integrating into the DET1 complex in a manner analogous to the endogenous protein and thus the myc-tDET1 transgene is likely to be an accurate proxy for the endogene.

3.3 Localisation of myc-tDET1

3.3.1 Introduction

A number of studies have now shown that DET1 is part of a nuclear localised complex involved in the control of light-regulated gene expression. However DET1 has no DNA binding activity, nor does it appear to interact with RNA polymerase II (G Benvenuto and C Bowler, unpublished observations), thus the means by which DET1 regulates gene expression is not clear. Previous work in the laboratory demonstrated that DET1 binds chromatin and that this interaction is mediated by a specific interaction with the N-terminal tail of histone H2B (Benvenuto et al., 2002). DET1 binding to chromatin provides a possible mechanism by which it could interact with light regulated genes for which it appears to act as a repressor (Chory et al., 1989) (Chory and Peto, 1990), although no evidence of DET1 association with light-regulated genes (or any other genomic element) has yet been demonstrated. In order to further our understanding into the mechanism of action of the DET1 complex, the major aim of this thesis is to confirm the interaction between DET1 and chromatin and to determine to which regions of the genome it localises.

3.3.2 Subnuclear localisation

Immunolocalisation was performed on fixed protoplasts from wild-type or myc-tDET1 transgenic lines using antibodies against myc, H3K4me2 (histone H3 di-methylated at lysine 4) and H3K9me2 (histone H3 di-methylated at lysine 9), the latter two being markers of heterochromatin and euchromatin, respectively. DNA was counterstained with DAPI, which labels predominantly heterochromatin. Image stacks were acquired and deconvolved using an Expectation Maximum algorithm (Conchello and McNally, 1996), and DAPI and antibody channels were overlaid.

Immunolocalisation using a myc-tDET1 transgenic line with an anti-myc antibody (Fig 3.7c) showed specific staining of the nucleus that was not present in the wild-type or no-antibody controls (data not shown). This result is consistent with previously published observations showing that DET1 is constitutively nuclear (Schroeder et al., 2002) see also Fig 3.4), and indicates that the fusion protein is able to localise to the correct subcellular compartment.
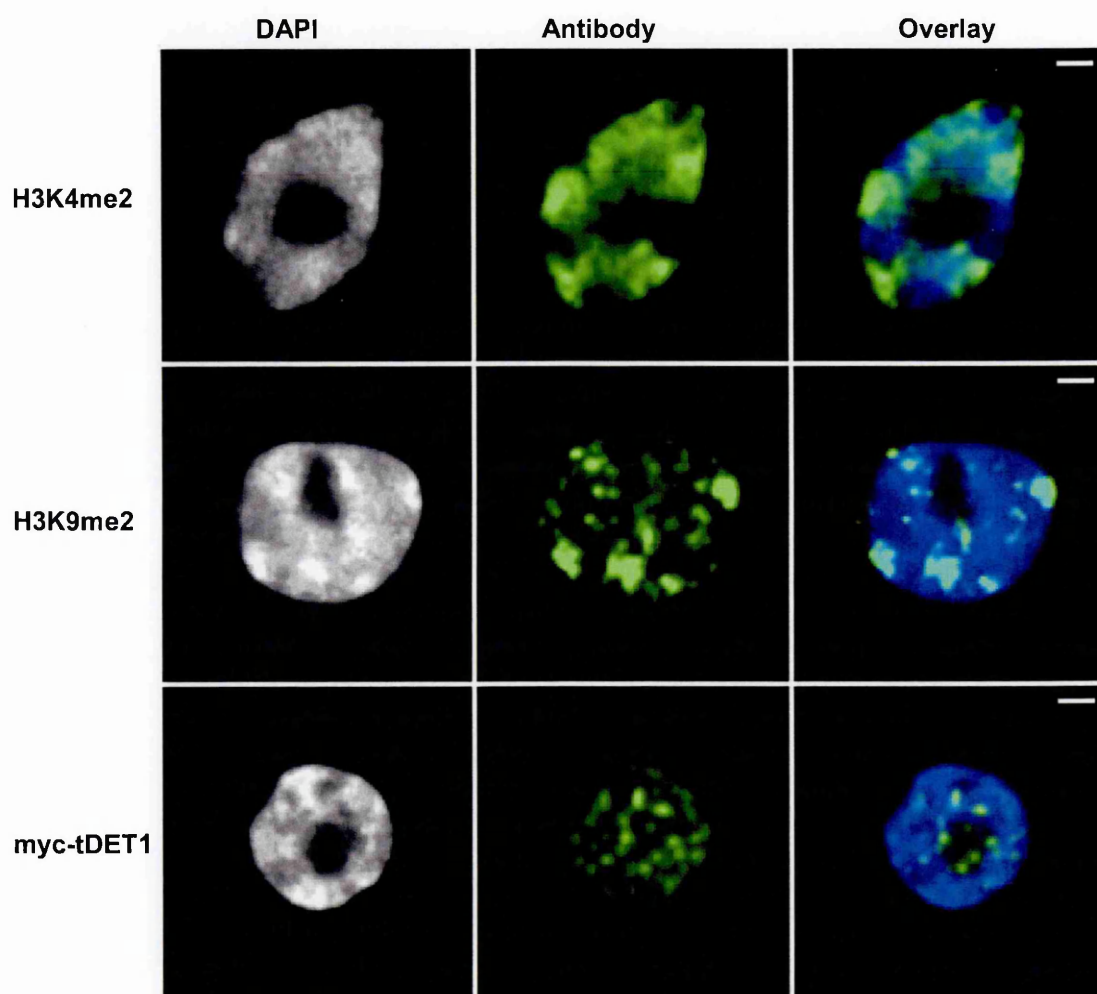
Figure 3.7 Immunolocalisation on *Arabidopsis* protoplasts. First column, DAPI staining of nuclei. Second column, Immunolabelling with primary antibody and Alexa 488-conjugated secondary antibody. Third column, merge of antibody and DAPI panels. First row: H3K4me2 is distributed throughout the euchromatin. Second row: H3K9me2 is restricted to heterochromatic knobs and small puncta within the euchromatic regions. Third row: myc-tDET1 shows a punctuate distribution throughout the euchromatin, with higher levels at foci around the nucleolus. White scale bar represents 1 µM.

DAPI staining of interphase Arabidopsis nuclei revealed 6-10 chromocenters which contain the centromeres and permanently condensed or "constitutive" heterochromatin ((Fransz et al., 2003), see Fig. 3.7). The nucleolus is visible as 1 (or 2) region(s) free from DAPI staining around which a few chromocenters are typically located. The remaining chromocenters are usually located around the periphery of the nucleus. The remaining area of weaker, rather uniform DAPI staining is termed euchromatin and contains the relatively decondensed, gene rich regions of the chromosomes. This microscopically visible euchromatin space consists of both "true" euchromatin consisting of genes that are actively transcribed and the "cryptic" heterochromatin consisting of genes which are transcriptionally silent but are inducible under certain conditions (Fransz et al., 2006).

Immunolocalisation with the anti-H3K9me2 (a marker of silenced chromatin) labels mainly the chromocenters while staining with anti-H3K4me2 (a marker of transcribed, decondensed chromatin) labels the euchromatic region in a rather even manner (Fig 3.7). Anti-myc staining (Fig 3.7) shows that myc-tDET1 is present mainly in the euchromatic regions of the nucleus and foci at the nuclear and nucleolar peripheries. However its distribution is much more punctuate than H3K4me2 suggesting that it may be localised to a limited number of genomic loci and that these in turn may form discrete nuclear structures that may correspond to cryptic heterochromatin. This pattern contrasts with that of chromatin remodelling proteins involved in the silencing of transposons and repetitive DNA such as DDM1 (Decrease in DNA Methylation 1, (Zemach et al., 2005), which localises to heterochromatin. Instead the pattern of myc-tDET1 localisation resembles that described for another repressor of transcribed genes, LHP1 (Like Heterochromatin Protein 1, (Libault et al., 2005), which is consistent with DET1 being a negative regulator of light activated genes.

3.3.3 Recruitment to chromatin

The finding that myc-tDET1 is recruited to specific chromatin territories, while supportive of a possible interaction with chromatin, could be due to other phenomena, for example The DET1 complex is known to interact with a number of other nuclear localised proteins which could potentially be responsible for recruitment to these regions. In order to differentiate between these two possibilities, protoplasts were permeablised with Triton-X 100 prior to fixation in order to wash out soluble proteins. This treatment had little effect on the localisation of myc-tDET1, (Fig 3.8) suggesting that it is retained in the nucleus by a strong interaction with chromatin.

No Triton-X 100 | **10 min Triton-X 100 treatment**

DAPI | Antibody | Overlay | DAPI | Antibody | Overlay

A, B, C
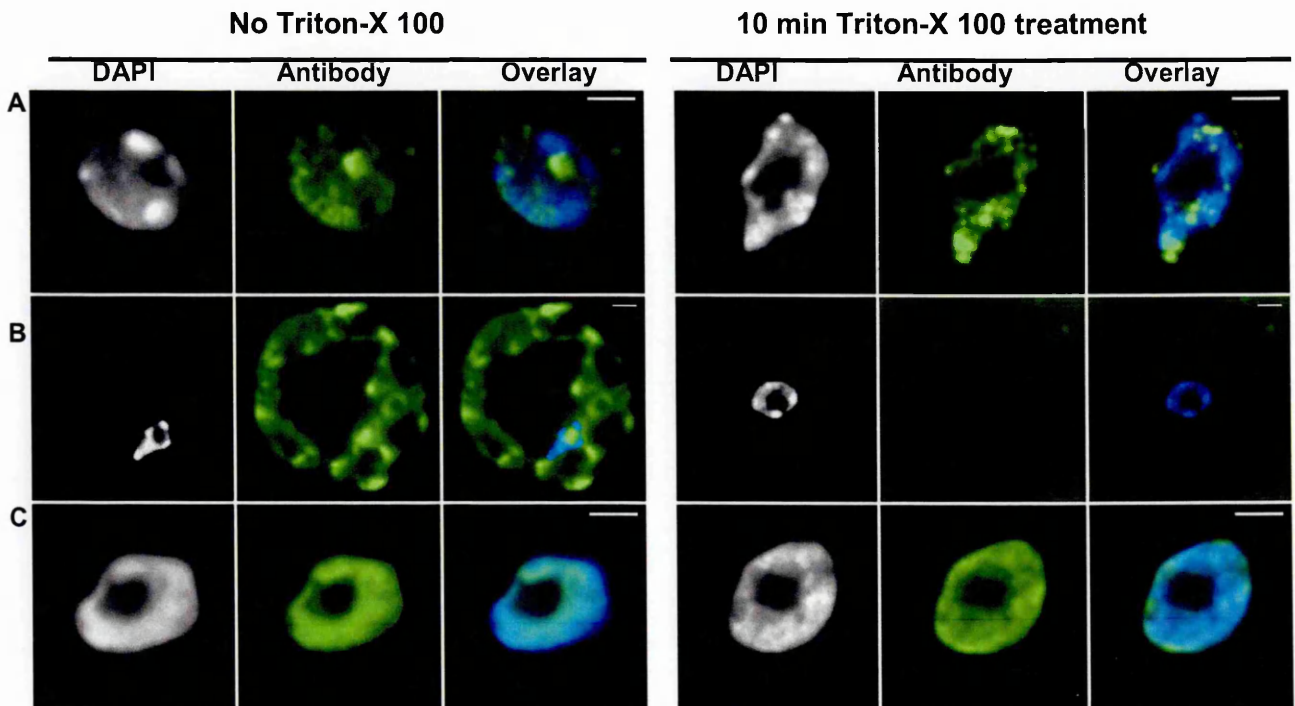
Figure 3.8 Immunolocalisation on Triton-X 100 treated Arabidopsis protoplasts shows that myc-tDET1 remains associated with chromatin in permeablised cells. Left: Control cells fixed without prior permeablization. Right: Protoplasts permeablised with Triton-X 100 prior to fixation. **(A)** myc-tDET1 is retained in the nucleus after Triton-X 100 permeablization. **(B)** YFP negative control is lost from the cell after Triton-X permeablization. **(C)** tH2A-YFP positive control is retained in the nucleus after Triton-X 100 permeablization. Scale bar represents 2 μM.

3.4 ChIP

3.4.1 Introduction

The results obtained above suggest that DET1 interacts directly with chromatin at restricted loci within the euchromatic regions of the nucleus. However, due to the limited resolution of fluorescence microscopy, this method is unable to provide more specific information. We thus decided to employ Chromatin Immunoprecipitation (ChIP) to obtain localisation information at the level of individual genomic loci (down to 1000 bp resolution). ChIP followed by semi-quantitative PCR should allow us to determine at what regions of the genome DET1 is binding and thus give us a better understanding of its mode of action.

3.4.2 Setup 1: crosslinking

A common problem encountered when performing Chromatin Immunoprecipitation experiments is that the antibody, even one that works well for conventional immunoprecipitation, may not function efficiently during the ChIP experiment.

Reasons for this can include masking/destruction of the epitope due to formaldehyde crosslinking or protein denaturation (of the epitope) due to the presence of 1% SDS in the sonication buffer. To this end it was necessary to confirm that myc-tDET1 can be efficiently immunoprecipitated from formaldehyde-fixed cells under the conditions used during the ChIP experiment. Three week old Arabidopsis seedlings expressing myc-tDET1 were cross-linked with 1% formaldehyde for 10 minutes and the ChIP experiment performed as usual except that after immunoprecipitation with the anti-myc antibody, the immunoprecipitated protein was eluted from the beads, run on an SDS-PAGE gel and subject to western blot analysis with an anti-myc antibody (a rabbit anti-myc antibody was used for the immunoprecipitation while a mouse anti-myc antibody was used for western blotting to avoid any problems with cross-reactivity). As shown in Fig 3.9a, myc-tDET1 was efficiently immunoprecipitated from the formaldehyde crosslinked material, thus indicating that this antibody and these experimental conditions are suitable for ChIP experiments.
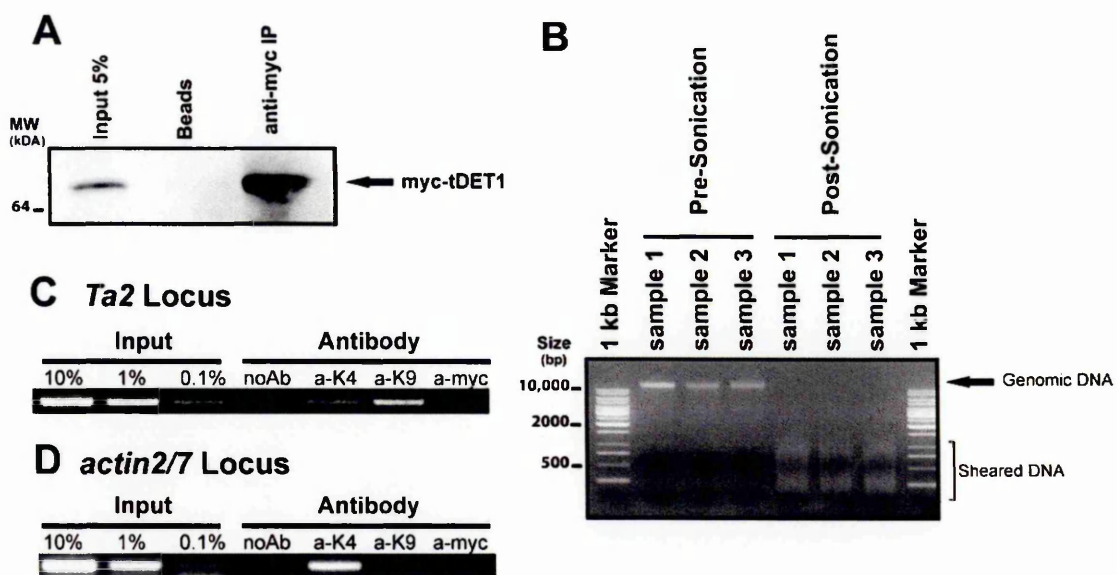
Figure 3.9 Chromatin Immunoprecipitation experiments preformed on myc-tDET1 expressing plants. **(A)** Western blot of immunoprecipitated material confirming that myc-tDET1 can be immunoprecipitated from formaldehyde-fixed Arabidopsis nuclei. **(B)** Agarose gel of DNA from formaldehyde fixed Arabidopsis nuclei before and after sonication confirming that the DNA has been sheared to between 2000 and 500 bp in length. **(C)** and **(D)** Semi-quantitative PCR to detect possible enrichment of the *Ta2* retrotransposon **(C)** or the *actin2/7* gene **(D)** in chromatin immunoprecipitated with antibodies against H3K4me2,H3K9me2, MYC or no antibody control.

3.4.3 Setup 2: Control Experiments

As Chromatin Immunoprecipitation can be a rather difficult technique to set up, preliminary experiments were performed to see if we could replicate previously

published findings (Tariq et al., 2003) and thus confirm that the chromatin immunoprecipitation method was working under our experimental conditions. Nuclei from formaldehyde-fixed Arabidopsis seedlings expressing myc-tDET1 were sonicated to shear the chromatin, a sample was taken, from which the DNA was purified and run on a gel to ensure that the chromatin had been sheared to the appropriate size range (Fig 3.9b). The sheared chromatin was then immunoprecipitated with antibodies against histone H3 di-methylated at lysine 4 (H3K4me2) or H3 di-methylated at lysine 9 (H3K9me2), ("active" and "inactive" chromatin modifications, respectively). DNA was purified from the immunoprecipitated chromatin and probed with primers for loci that are known to be enriched in these modifications. Primers against the *Ta2* retro-transposon showed specific enrichment in the H3K9me2 immunoprecipitation (Fig 3.9c) and a low level of enrichment of H3K4me2 while primers against the *actin2/7* gene showed enrichment in the H3K4me2 at this locus (Fig 3.9d). These results are consistent with previously published reports (Gendrel et al., 2002; Tariq et al., 2003), and indicated that the chromatin immunoprecipitation experiment was successful under our experimental conditions.

3.4.4 **Identification of DET1 targets**

Chromatin immunoprecipitation was repeated using material from the myc-tDET1 line and a rabbit anti-myc antibody previously shown to efficiently immunoprecipitate myc-tDET1 from formaldehyde-fixed nuclear extracts (Fig 3.9a). Initial experiments with primers against *Ta2* failed to show any enrichment in the anti-MYC immunoprecipitation (Fig 3.9c). These data are in agreement with the finding that myc-tDET1 did not localise to heterochromatin regions in the immunofluorescence experiments (Fig 3.7). Furthermore, primers against *actin2/7* (an actively transcribed gene) failed to show enrichment in the anti-myc immunoprecipitation (Fig 3.9d). This result is also expected, as DET1 is a putative transcriptional repressor and we would not expect it to be present at an active gene such as *actin2/7*.

The phenotype of the *det1* mutant infers that *DET1* encodes a negative regulator of light-inducible gene expression (Chory et al., 1989). In order to identify possible DET1 targets we analysed the literature for light-induced genes that might be directly regulated by DET1. Although approximately 8,000 genes are misregulated in dark-grown *det1* mutants when compared to wild-type (Ma et al., 2003), only a small portion of these are likely to be under direct control of DET1. We decided to focus on *CAB2* (chlorophyll-A/B binding protein 2) and *HEMA1* (heme-oxygenase 1), two

genes whose light regulated expression has been well studied (McCormac and Terry, 2002) and are also known to be misregulated in the *det1* mutant background (Mayer et al., 1996). In addition the *CAB2* promoter contains the DET1 dark response element (DtRE, (Maxwell et al., 2003)), a 40-bp element required for the repression of *CAB2* in a DET1-dependent manner, that may serve as a targeting signal for the DET1 complex.

While the most striking aspect of the *det1* mutant is its de-etiolated phenotype and its inability to repress light-induced genes in the dark, the *det1* mutant also has a strong phenotype when grown in the light. Previous studies have shown that *DET1* expression levels remain relatively constant throughout the life-cycle of Arabidopsis and are not controlled by light (Pepper et al., 1994). The DET1 protein also appears to remain constitutively nuclear localised throughout the lifecycle of the plant (Schroeder et al., 2002). This is in contrast to its putative interacting partner COP1, which is exported to the cytosol in response to light (von Arnim and Deng, 1994). These data together may suggest that DET1 has an important role in light-grown plants.

3.4.5 CAB2

ChIP was performed on formaldehyde-fixed, 4-day old dark-grown myc-tDET1 seedlings using an antibody against MYC. Primers against the *CAB2* promoter detected enrichment of this locus in chromatin co-immunoprecipitated with myc-tDET1 (Fig 3.10b). When the experiment was repeated on formaldehyde-fixed light grown plants no enrichment of this locus could be detected from the myc-tDET1 immunoprecipitation (Fig 3.10c), nor could enrichment be detected in material from dark-grown wild-type plants (Fig 3.10a). This results indicates that myc-tDET1 is present at (or near) the *CAB2* promoter during skotomorphogenic growth when this gene is not expressed. In contrast, myc-tDET1 appears to be absent from the *CAB2* promoter in light-grown seedlings, when the *CAB2* gene is expressed.
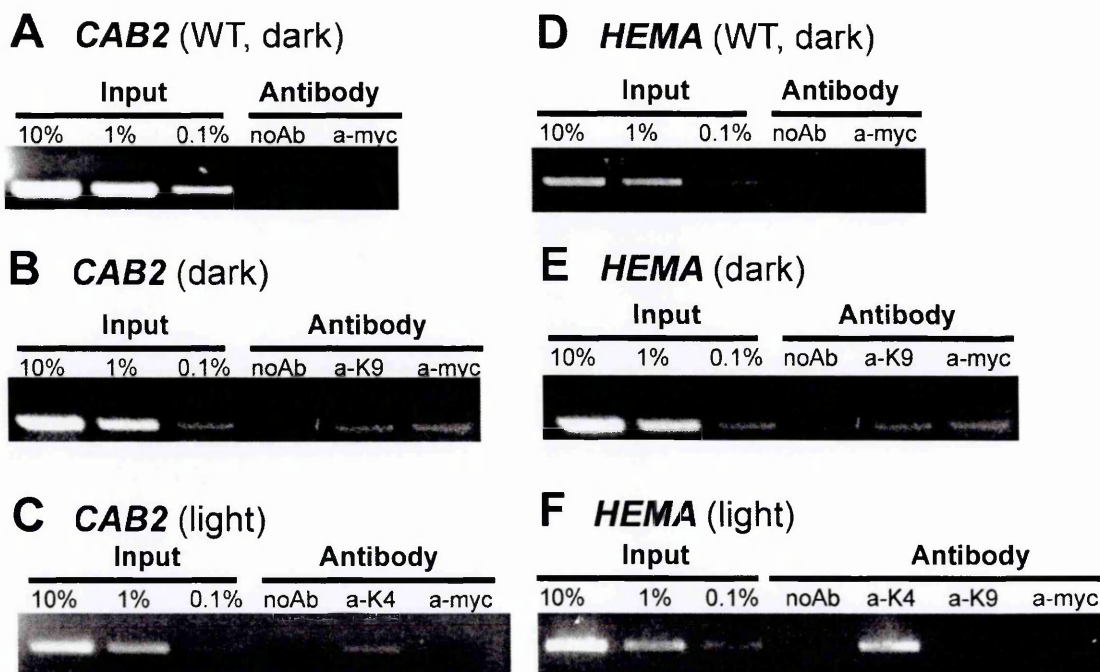
A *CAB2* (WT, dark)

B *CAB2* (dark)

C *CAB2* (light)

D *HEMA* (WT, dark)

E *HEMA* (dark)

F *HEMA* (light)

Figure 3.10 Semi-quantitative PCR to detect possible enrichment of the *CAB2* and *HEMA1* promoters in chromatin immunoprecipitated with antibodies against H3K4me2, H3K9me2 or MYC. **(A)** Chromatin immunoprecipitated from dark-grown wild type plants is assayed for enrichment of *CAB2*. **(B)** Chromatin immunoprecipitated from dark-grown myc-tDET1 plants is assayed for enrichment of *CAB2* **(C)** Chromatin immunoprecipitated from light-grown myc-tDET1 plants is assayed for enrichment of *CAB2*. **(D)** Chromatin immunoprecipitated from dark-grown wild type plants is assayed for enrichment of *HEMA1*. **(E)** Chromatin immunoprecipitated from dark-grown myc-tDET1 plants is assayed for enrichment of *HEMA1*. **(F)** Chromatin immunoprecipitated from light-grown myc-tDET1 plants is assayed for enrichment of *HEMA1*.

The primers against the *CAB2* promoter also detected enrichment of this locus in chromatin immunoprecipitated with the H3K9me2 antibody from dark-grown seedlings (Fig 3.10b). In contrast no enrichment of the *CAB2* promoter was detected in chromatin immunoprecipitated with the anti-H3K9me2 antibody in light-grown seedlings (data not shown). This result was as expected because it is known that the *CAB2* gene is not transcribed under these (skotomorphogenic) conditions (McCormac and Terry, 2002) and H3K9me2 is a typical marker not only of constitutive heterochromatin but also of transcriptionally silent genes (cryptic heterochromatin) in Arabidopsis (Gendrel et al., 2002). This can be seen in Fig 3.7b where low levels of H3K9me2 decorating the "cryptic" heterochromatin are also found in the euchromatin compartment.

3.4.6 HEMA1

When the immunoprecipitated chromatin from dark and light grown seedlings was probed with primers against the *HEMA1* promoter I found enrichment of this locus in chromatin co-immunoprecipitated with myc-tDET1 from dark-grown but not light-grown seedlings (Fig 3.10e, f). Likewise this locus was enriched in H3K9me2-containing chromatin from dark-grown but not light grown seedlings. These results indicate that, like for *CAB2*, myc-tDET1 and H3K9me2 are present at the *HEMA1* promoter during skotomorphogenic growth when this gene is repressed and that they are absent from this promoter in the light when *HEMA1* is expressed.

These results provide the first direct evidence that DET1 controls light-regulated gene expression by directly interacting with the genes themselves (or their regulatory elements). This finding is quite novel because although it has long been known that DET1 was involved in light signalling pathways and light-regulated gene expression (Chory et al., 1989; Chory and Peto, 1990) (Pepper et al., 1994) it was (and still is) generally assumed that DET1 functions as part of a nuclear localised E3 ubiquitin-ligase complex that degrades light signalling intermediates such as phytochromes and positive regulators of light signalling such as the HY5, HYH and LAF transcription factors (Jang et al., 2005; Seo et al., 2004; Seo et al., 2003). These data suggest that if this is indeed its mechanism of action, then it may occur directly at the promoters that these transcription factors are targeted to. Alternatively DET1 may repress these genes by a more direct mechanism such as chromatin remodelling in which it would modify the chromatin around these genes (e.g., by histone ubiquitination) in such a way that the gene becomes refractory to activation. These mechanisms do not preclude each other and DET1 may function by a combination of both mechanisms.

3.4.7 POR-A

The previous data established that DET1 interacted with the promoters of two light induced genes, *CAB2* and *HEMA1,* in the dark. Given that the *det1* mutant displays a strong phenotype in the light we hypothesised that in the light it might equally interact with the promoters of light repressed genes. Analysis of the literature suggested that *POR-A* (NADPH:protochlorophyllide oxidoreductase-A; (Armstrong et al., 1995) is strongly down-regulated during the transition from skotomorphogenic to photomorphogenic growth and might be a potential DET1 target.

Primers that amplify the promoter of *POR-A* were designed and used to assay for enrichment of this locus in DNA from 4-day-old dark grown or light grown plants. Enrichment of myc-tDET1 was detected at the promoter of *POR-A* in material from light-grown, but not dark grown myc-tDET1 seedlings (Fig 3.11a, b). As a further control to ensure these results were not due to an aspecific interaction with the antibody, we repeated the experiment on light-grown wild-type plants. In this case we detected no enrichment of the *POR-A* promoter in the sample immunoprecipitated with the anti-myc antibody (Fig 3.11c).

This result suggests that myc-tDET1 indeed interacts with the *POR-A* promoter and is not just some artefact caused by the antibody acting in an aspecific manner. The finding that DET1 localises to the *POR-A* promoter suggests that DET1 has a wider role in light-regulated gene expression. In addition this result suggests that DET1 is required for correct gene-regulation in light grown plants and may in part explain the persistence of a strong phenotype in light-grown *det1* mutants.
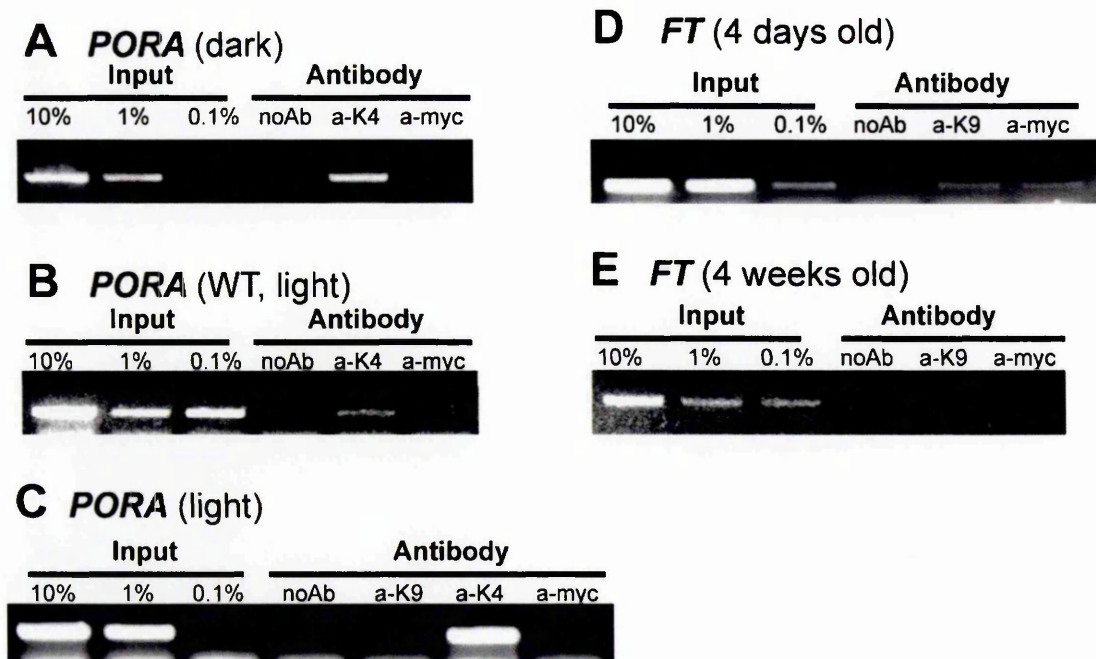
Figure 3.11 Semi-quantitative PCR to detect possible enrichment of the *POR-A* and *FT* promoters in chromatin immunoprecipitated with antibodies against H3K4me2, H3K9me2 or myc. **(A)** Chromatin immunoprecipitated from dark-grown myc-tDET1 plants is assayed for enrichment of *POR-A*. **(B)** Chromatin immunoprecipitated from light-grown wild-type plants is assayed for enrichment of *POR-A*. **(C)** Chromatin immunoprecipitated from light-grown myc-tDET1 plants is assayed for enrichment of *POR-A*. **(D)** Chromatin immunoprecipitated from 4-day-old dark-grown myc-tDET1 plants is assayed for enrichment of *FT*. **(E)** Chromatin immunoprecipitated from 4 week old myc-tDET1 plants is assayed for enrichment of *FT*.

3.4.8 Flowering locus-T

The most striking aspect of the *det1* mutant is its inability to maintain etiolated /skotomorphogenic growth in the absence of light, hence its original description as a negative regulator of plant photomorphogenesis. However the *det1* mutant displays many other phenotypic abnormalities, including reduced apical dominance, day-length insensitive early flowering, abnormal flower development (resulting in partial male sterility) and aberrant leaf morphology (Chory et al., 1989; Chory and Peto, 1990; Pepper et al., 1994; Pepper and Chory, 1997). These phenotypes are indicative of defective regulation of the pathways controlling plant development and suggest that DET1 may also be involved in regulating the expression of genes controlling plant development. As the *det1* mutant appears to have defects in the regulation of flowering-time (Pepper and Chory, 1997), we decided to see if DET1 interacts with the *Flowering Locus-T* (*FT*) gene. *FT* is a strong promoter of flowering that is up regulated in response to growth in long-day conditions and promotes the vegetative-to-floral transition (Kardailsky et al., 1999; Kobayashi et al., 1999). Under vegetative growth its expression is strongly repressed by a pathway controled by the phytochrome photoreceptors (Devlin et al., 2003, Halliday, 2003 #399). Abolition of this repression, for example in the *phyB* mutant or the *lhp1* (like-heterochromatin-protein 1) mutant, a repressor of many developmentally regulated genes, results in early flowering regardless of day length (Gaudin et al., 2001) (Kotake et al., 2003).

Primers that amplify the *Flowering Locus-T* (*FT*) promoter were designed and used to assay chromatin immunoprecipitated from 4-day-old seedlings. Enrichment of this locus was detected in chromatin co-immunoprecipitated with myc-tDET1, indicating that myc-tDET is present at the *FT* promoter (Fig 3. 11d) under these conditions. Enrichment of H3K9me2 at the *FT* promoter was also detected, consistent with FT not being expressed in 4-day-old seedlings (Kotake et al., 2003). In this case the presence of H3K9me at the FT promoter is particularly interesting as H3K9me recruits LHP1 via its chromodomain (Jackson et al., 2002) and LHP1 is in turn essential for repression of *FT* (Kotake et al., 2003).

The presence of myc-tDET1 at the *FT* promoter suggests that DET1 may directly repress this promoter and extends the number of loci at which we have found myc-tDET to four. *FT* is the only one of these loci not directly regulated by light (although it is regulated by day length in a *CONSTANS* (CO)-dependent manner (Kardailsky et

al., 1999; Kobayashi et al., 1999). This suggests that DET1 may have a more general role in transcriptional regulation as opposed to just being involved in light-dependent gene expression. This finding is not unexpected, because a DET1 homolog in a non-photosynthetic organism, the *Drosophila* ABO protein, has previously been shown to bind chromatin and to act as a transcriptional repressor (Berloco et al., 2001). The presence of DET1 at the promoter of *FT*, a key regulator of plant development, suggests that DET1 may directly regulate the expression of genes controlling plant development. This would explain the highly pleiotropic nature of light-grown *det1* mutants whose phenotype cannot be attributed to impaired light signalling alone (Chory and Peto, 1990; Mayer et al., 1996). The finding that myc-tDET1 (and thus presumably the DET1 complex) is localised to the *FT* promoter is particularly intriguing because previous studies have shown that LHP1 is required for the repression of this promoter (Kotake et al., 2003). While in fission yeast (*Schizosacharomyces pombe*) a Cul4-containing complex is responsible for H3K9 dimethylation at certain developmentally regulated loci (Jia et al., 2005), this in turn recruits the *S. pombe* LHP1 homolog, Swi6, which is required for maintenance of this repression. Our finding that both the DET1 complex and H3K9me2 are present at a *LHP1*-repressed promoter suggest that a similar mechanism may be present in Arabidopsis and suggests a possible mode of action for the putative transcriptional repressive activity of the DET1 complex.

In summary these results suggest that DET1 is present together with H3K9me2 at the promoters of light induced and light repressed genes only when they are in their repressed state. DET1 and H3k9me2 may also localise to the promoters of genes involved in plant development when they are in their repressed state. While the Chromatin Immunoprecipitation data show that myc-tDET1 colocalizes with H3K9me2 at each of these four loci, it is obvious that the relationship between these two features is not exclusive and is likely to be restricted to a subset of loci, as demonstrated by the presence of H3K9me2 but not myc-tDET1 at the *Ta2* retrotransposon. Conversely, the immunolocalisation data do not show a strong correlation between myc-tDET1 and H3K9me2 localisation. Thus any relationship between DET1 and HeK9me2 is likely to be complex.

3.4.9 H3K4me2

H3K4me2 is a marker of active genes in metazoans (Schneider et al., 2004; Schubeler et al., 2004), and consistent with this we found it to be distributed throughout the euchromatic regions of the nucleus (Fig3.7a), and enriched at the

active *actin2/7* gene but present at very low levels at the transcriptionally silent *Ta2* retrotransposon (Fig3.9c,d). However in further experiments we consistently found H3K4me2 at the promoters of genes known to be silent and also enriched in H3K9me2 (data not shown and Fig 3.11c).

One possibility is that this antibody is not specific and is recognising unmodified H3 or another epitope found in silent chromatin, but this is not likely to be the sole reason because the *Ta2* retrotransposon was not highly enriched in chromatin immunoprecipitated with this antibody (Fig 3.9c). Instead it appears that in Arabidopsis H3K4me2 is present in all genes and their promoters irrespective of whether they are silent or active. This feature may be associated with RNApol-II because the *Ta2* retrotransposon (which is probably transcribed by RNApol-IV) is largely devoid of this mark (Fig 3.9c). While this study was underway another group (Alvarez-Venegas and Avramova, 2005) demonstrated that H3K9me2 was present in all the genes they examined, whether they were expressed or not, but was absent from intergenic regions.

4 Discussion

4.1 Overview

The results presented in this thesis describe the construction and testing of a modified Gateway cloning system designed to facilitate the generation of transgenic plants overexpressing proteins tagged with a variety of epitope tags.

As part of a larger study into light regulated gene expression, I investigated the negative photomorphogenesis regulator DET1. An interaction between the DET1 complex and the CUL4 ubiquitin ligase was demonstrated by co-immunoprecipitation using a line expressing myc-tDET1. Immunolocalisation experiments suggested that DET1 localizes to discrete foci in the nucleus that may correspond to cryptic heterochromatin (silent genes). In agreement with this, chromatin immunoprecipitation demonstrated that DET1 was localised to the promoter of several light-regulated genes specifically under conditions where they were repressed.

4.2 Modified Gateway Cloning System

In this thesis the creation of a modified Gateway cloning system is described. The major characteristic of this system is that the epitope tag is contained within the Entry cassette instead of in the Destination vector. This results in the entire open-reading-frame (ORF) encoding the epitope-tagged protein of interest being contained within the Entry vector so that it can be subsequently recombined into a plant binary Destination vector containing a promoter (35S or XVE) and resistance cassette (kanamycin, hygromycin or Basta) of choice without concern over reading frame. The resulting vectors were used for transformation of Arabidopsis by the floral dip method (this thesis) or for transient *A. tumefaciens*-mediated transformation of *Nicoitiana benthamiana* (M. Ron and G. Benvenuto, unpublished data). These cassettes can also be recombined into minimal overexpression vectors optimized for transient transformation of plant cells/protoplasts by biolistic bombardment or poly-ethylene-glycol (PEG)-mediated transformation of plant protoplasts. Alternatively the tag-protein fusion cassette can be recombined into vectors for *in vitro* expression or overexpression in bacterial, yeast, insect cell or mammalian cell systems.

The design of this system overcomes a number of weaknesses of the traditional Gateway system, such as the presence of the *att* recombination site between the tag

and protein of interest, which results in a long (typically 15-20 amino acids), often highly charged, sequence due to the *att* sequence and polylinker (Parr and Ball, 2003). This may result in a non-functional or insoluble protein-tag fusion. A further problem is that the long polylinker renders fluorescent protein fusions unsuitable for FRET studies because the long polylinker allows too much freedom of movement between the protein and the tag, making FRET measurements unreliable (Periasamy and Day, 1999). The modified cloning system also allows the use of a number of tags that are not available in conventional Gateway plant binary vectors and furthermore allows them to be recombined into vectors with a variety of promoters or selectable markers, thus allowing many more options when performing experiments.

Recently an alternative methodology to solve this problem was described by Morlin and colleagues (Colwill et al., 2006). In their system, a mammalian intron splice donor site is placed immediately downstream of the N-terminal tag in the Destination vector and a splice acceptor site placed immediately upstream of the multiple cloning site in the Entry vector. When the two vectors are recombined this results in the *att* sequence being contained within an intron, which is spliced out of the pre-mRNA (Colwill et al., 2006). Although elegant, limitations of this system include its use being restricted to organisms that can process mammalian splicing signals and the need to introduce the splice sequences into both the Entry and the Destination vectors.

In this thesis, the construction of 12 different Entry cassettes, each with an amino- or carboxy-terminal tag for 6xmyc, 3xHA, Strep, GST, EYFP or ECFP is described. A number of proteins currently under investigation in the laboratory, tDET1, tDDB1, tDDB2, tUbiqutin, tH2A and tH2B were cloned into these Entry cassettes, recombined into Destination vectors with the desired promoter and resistance cassettes, and used for transformation of *A. thaliana*.

Homozygous lines were obtained that expressed fusion proteins of the correct molecular weight for both amino and carboxy terminal fusions for a number of these proteins tagged with a number of different tags (Table 3.1). These results confirm that the modified Gateway cassettes constitute an efficient and reliable method for expression of transgenes with a variety of tags, promoters and selectable markers. Interestingly, while lines expressing tagged H2A, H2B and Ubiquitin fusions were readily obtained, this was not the case for DDB2 and DET1 fusions, which were not detectable under our experimental conditions when expressed from a 35S promoter. However EYFP-tDET1 was detected when transiently expressed from the inducible

XVE promoter (Fig 3.4a). A possible reason for this is that the genes in the constitutively expressed lines are silenced (which has previously been observed for myc-tDET1 expressed from a 35S promoter in tomato; (Davuluri et al., 2004). A simpler explanation is that the difference observed is simply due to the XVE promoter being stronger than the 35S promoter; previous studies have suggested that the XVE promoter can produce up to 8 times more transcript than the 35S promoter (Zuo et al., 2000).

4.3 Characterisation of transgenic Myc-tDET1 Arabidopsis lines

Because it has not yet been possible to generate an antibody against DET1, it was necessary to generate an epitope tagged DET1 construct for expression in Arabidopsis. Homozygous transgenic Arabidopsis lines expressing myc-tDET1 under control of a 35S promoter were therefore obtained.

Data from both plants and mammals indicate that DET1 is part of a multiprotein complex together with DDB1, COP10 and CUL4 (Schroeder et al., 2002) (Wertz et al., 2004; Yanagawa et al., 2004). Subsequently, in the host laboratory it was shown that the myc-tDET1 construct that I transferred into Arabidopsis can be used to co-immunoprecipitate DDB1. I have now extended this finding to demonstrate that an anti-Cul4 serum can be used to co-immunoprecipitate myc-tDET1. This result was confirmed using GST-pulldown assays and yeast-2-hybrid analysis by our collaborators (Bernhardt et al., 2006). Together these results suggest that in plants a core-complex of COP10, DET1 and DDB1 forms a substrate adaptor for CUL4 (Fig 4.1). The interaction with CUL4 is probably dependent on the neddylation of CUL4 (Cope et al., 2002; del Pozo et al., 2002). The DET1 complex is also likely to interact with a number of other proteins/complexes. For example, DET1 binds chromatin (Benvenuto et al., 2002), OsDDB1 (rice) binds OsDDB2 (Ishibashi et al., 2003), while COP10 (and possibly also DET1) interacts with COP1 (Suzuki et al., 2002) which is in a separate 700 kDa complex (Saijo et al., 2003). These interactions suggest that the DET1 complex is likely to be involved in a number of different functions, each of which is likely to require interaction with different accessory factors. Consequently, the complement of proteins interacting with the DET1 complex is probably heterogeneous and may vary with cell cycle, developmental stage and cell type.
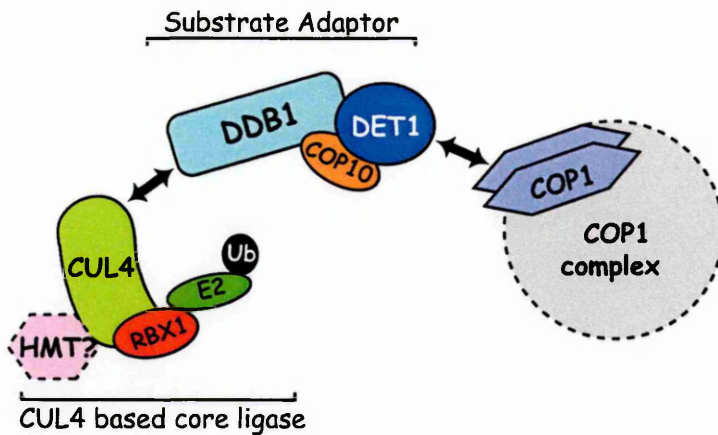
Fig 4.1 Schematic of the DET1 complex. DET1, DDB1 and COP10 form the DET1 core complex, which acts as the substrate adaptor for a CUL4-based ubiquitin ligase, which contains CUL4, the adaptor protein RBX1, and a ubiquitin E2 ligase. The presence of a histone methyltransferase in the complex is speculative and has not yet been demonstrated in plants.

4.4 Localisation of myc-tDET1

Immunofluorescence experiments demonstrated that the myc-tDET1 fusion protein was exclusively nuclear localized (Fig 3.7), which is in agreement with previously published data that show that EGFP-AtDET1 is nuclear localized and can complement the *det1-1* mutation (Schroeder et al., 2002), as well as our data from the EYFP-tDET1 fusion protein expressed from the XVE promoter (Fig 3.4a).

The myc-tDET1 immunofluorescence signal displayed a punctate distribution throughout the euchromatic (gene-rich) regions of the nucleus, with higher concentrations around the nuclear periphery and around the nucleolus. Like H3K4me2 (a marker for transcribed genes), myc-tDET1 appears to be localised throughout the euchromatic regions of the nucleus. However, while H3K4me2 is distributed relatively evenly throughout the euchromatin, myc-tDET1 distribution was more punctate, often with foci at the nuclear and nucleolar peripheries. This suggests that myc-tDET1 is localised to a limited number of genomic loci that form discrete nuclear structures. While the bulk of H3K9me2 (a marker of silent chromatin) is present in the intensely DAPI-stained chromocenters which contain the constitutive heterochromatin, lower levels are also found in small foci thorough the euchromatic regions of the nucleus, which probably corresponds to cryptic heterochromatin (Fransz et al., 2006). These two H3K9me2 populations appear to be deposited by different histone methyltransferases, as the *kyp* (*KYRYPTONITE*) mutant abolishes

H3K9me2 in the constitutive heterochromatin without affecting H3K9me2 in the cryptic heterochromatin (Jasencakova et al., 2003).

The distribution of myc-tDET1 appears similar to that of "cryptic" H3K9me2 (H3K9me2 present at foci outside of the chromocenters). Triton-X 100 treatment prior to fixation had little effect on the localization of myc-tDET1, suggesting that it is retained in the nucleus by a strong interaction with the chromatin. The localisation pattern of myc-tDET1 contrasts with that of most other Arabidopsis chromatin interacting proteins, such as methyl-CpG binding domain proteins, DDM1 (Decrease in DNA Methylation 1), or histone methyltransferases, all of which localize primarily to the chromocenters (Zemach et al., 2005; Naumann et al., 2005). Instead, the pattern of myc-tDET1 localisation resembles that described for the H3K9me2 binding protein LHP1 (Like Heterochromatin Protein 1), which has been shown to localize to foci within the euchromatin (Libault et al., 2005). Interestingly LHP1 has been shown to be necessary for the repression of transcribed genes, which contrasts with its proposed activity at constitutively silent loci in animal cells (Nakahigashi et al., 2005; Sung et al., 2006). This is consistent with its localisation to what appear to be "cryptic" and not "constitutive" heterochromatin.

In summary the territory occupied by myc-tDET1 appears to coincide with "cryptic" heterochromatin, as defined by H3K9me2 staining. Double immunolocalisation should be performed to verify this. In any case, this result is consistent with the hypothesis that DET1 acts as a negative regulator of gene expression by directly binding the promoters of genes that it represses. Interestingly, this localization pattern was also exhibited by LHP1, a protein which is also thought to repress transcribed genes by binding directly to their promoters (Libault et al., 2005).

4.5 Chromatin Immunoprecipitation

While the immunofluorescence data is supportive of the hypothesis that DET1 binds to specific genomic loci, confirmation of this required a higher resolution method such as chromatin immunoprecipitation. I therefore set up this technique in the laboratory, first using antibodies against specific histone modifications (H3K9me2 and H3K4me2) and known genomic targets (*Ta2* and *actin2/7*) within heterochromatin and euchromatin.

The constitutively silent *Ta2* retrotransposon is located within the pericentromeric region of chromosome 1 (Johnson et al., 2002), and during interphase it is located within a chromocentre (Soppe et al., 2002). In agreement with this I found high levels of H3K9me2 (a marker of constitutive heterochromatin) at this locus, but no myc-tDET1, consistent with its failure to localize to chromocenters in the immunofluorescence experiments (Fig 3.9). The constitutively expressed *actin2/7* gene had high levels of H3K4me2, a typical marker of expressed genes, but no myc-tDET1 was detected at this locus, which is consistent with the hypothesis that DET1 binds the promoters of only silent genes.

In etiolated (dark grown) seedlings H3K9me2 and myc-tDET1 were enriched at the promoters of *CAB2* and *HEMA1*, two light regulated genes known to be repressed in etiolated seedlings. The enrichment of H3K9me2 and myc-tDET1 was not detected in light-grown seedlings, where these two genes are active (Fig 3.10). This result provides the first direct evidence that DET1 binds to specific genomic loci. It is reasonable to assume that the binding is specific as we failed to detect binding to a number of other loci such as the Ta2 retro-transposon and the actin2/7 gene, nor was myc-tDET1 binding detectable at the *CAB2* and *HEMA1* loci when these two genes are expressed in light-grown plants.

Genetic experiments have previously defined DET1 as a negative regulator of *CAB2*, *HEMA1* and a number of other light-induced genes (Chory et al., 1989; Chory and Peto, 1990; Mayer et al., 1996; McCormac and Terry, 2002). However, the molecular mechanism by which DET1 acts is unknown and it has generally been assumed that it mediates repression of light-regulated gene expression by regulating the abundance of positive regulators of light signalling such as HY5 (McCormac and Terry, 2002; Pepper and Chory, 1997; Quail, 2002b). While the results presented here are not inconsistent with such a mechanism, they suggest that in the dark DET1 represses light-induced genes by directly binding to their promoters and maintaining them in a repressed state through an as yet unknown mechanism.

Given the highly pleiotropic phenotype of the *det1* mutant it has been suggested that DET1 may have a more general role in the regulation of signalling pathways and plant development (Mayer et al., 1996), rather than being a specific negative regulator of light-induced genes. In order to determine whether DET1 acts only to repress light induced genes in the dark or if it has a more general role, its interaction with the promoter of a gene known to be down-regulated by light, *POR-A*

(NADPH:protochlorophyllide oxidoreductase-A; Armstrong et al., 1995), was examined. Enrichment of myc-tDET1 was detected on the *POR-A* gene in light-grown myc-tDET1 expressing seedlings but not in wild-type or in dark-grown myc-tDET1 seedlings. Enrichment of H3K9me2 was also detected at the *POR-A* promoter in light-grown but not dark-grown seedlings, which is in agreement with earlier results showing that *POR-A* is only expressed in dark grown seedlings (Armstrong et al., 1995). Together these data establish that myc-tDET1 is present along with H3K9me2 at the promoters of several light-regulated genes when they are in a repressed or inactive state. It may therefore be a component of the "cryptic" heterochromatin visible by immunofluorescence microscopy.

The co-occurrence of myc-tDET1 and H3K9me2 at these loci is interesting because in the fission yeast *S. pombe* a complex containing homologs of Cul4 and DDB1 has been shown to silence several loci (including the telomeric and mating-type loci) (Horn et al., 2005; Jia et al., 2005). This complex functions by recruiting the H3K9-methyltransferase Clr4 which is responsible for high levels of H3K9me at these loci. H3K9me in turn recruits Swi6, the fission yeast homolog of heterochromatin-protein 1 (HP1), which maintains silencing by an as yet unknown mechanism (Li et al., 2005). In plants DET1, DDB1 and Cul4 interact (this thesis;(Bernhart et al., Submitted) (Schroeder et al., 2002). Plants also have 10 putative SU(VAR)3-9 (H3K9-specific) histone methyltransferases and a single Swi6/HP1 homolog, Like-Heterochromatin-Protein 1, HP1 proteins are normally localized to constitutive heterochromatin in most organisms, but in contrast plant LHP1 was shown to localise exclusively to punctate regions within euchromatin, probably corresponding to "cryptic" heterochromatin (Libault et al., 2005) where it acts as a negative regulator of several genes involved in developmental regulation (Nakahigashi et al., 2005). *tfl2* (*terminal-flower-2)* was originally described as an early flowering mutant (Larsson et al., 1998) and *TFL2* was subsequently shown to encode LHP1 (Kotake et al., 2003). *LHP1* was shown to antagonize activation of *Flowering Locus-T* (*FT*, a strong promoter of plant flowering) by *CONSTANS* and thus to prevent early flowering (Takada and Goto, 2003). Given that a Cul4-containing complex acts together with HP1 to repress certain loci in fission yeast it was therefore decided to investigate whether an analogous mechanism occurs in plants and whether myc-tDET1 was present at the *LHP1* repressed *FT* locus.

FT expression is detectable 6-12 days after germination in long-day grown plants (Kobayashi et al., 1999). Chromatin immunoprecipitation experiments showed that myc-tDET1 and H3K9me2 were indeed present at the *FT* locus in 4-day old plants, when *FT* is repressed, but not in 4 week old plants, when *FT* is expressed. Although to date the interaction between *LHP1* and *FT* has only been demonstrated genetically, the presence of myc-tDET1 at the *FT* promoter suggests that a silencing pathway similar to that found in *S. pombe* may exist in plants, where the DET1 complex would act to recruit LHP1, possibly via a Cul4 associated histone-methyltransferase.

In summary the results presented in this thesis suggest that DET1 is present at the promoters of some light induced and light repressed genes and also a gene controlling plant development (*FT*). In each case myc-tDET1 was present only when the gene was in its repressed state and its presence correlated with that of H3K9me2. The co-occurrence of myc-tDET1 and H3K9me2 was not absolute, as only H3K9me2 was detected at the constitutively silenced *Ta2* retrotransposon. Likewise, myc-tDET1 did not localise to the H3K9me2-rich chromocenters containing constitutive heterochromatin.

The distribution of H3K4me2, a mark normally associated with euchromatin (Jenuwein and Allis, 2001) was also examined by chromatin immunoprecipitation. As expected, H3K4me2 was present at high levels at a number of actively transcribed loci and present only at low levels at the constitutively silent *Ta2* retrotransposon. However, to our surprise H3K4me2 was also detected at a number of repressed loci that also contained H3K9me2, a typical repressive mark. These results suggest that while H3K4me2 is largely excluded from constitutive heterochromatin, in plants (in contrast to metazoans) it is present within the cryptic heterochromatin containing conditionally silent genes. Similar results were recently published by another group, who suggested that only tri-methylation (H3K4me3) and not di-methylation correlated with transcription in Arabidopsis (Alvarez-Venegas and Avramova, 2005). These results are consistent with recent findings that some marks are differently distributed between plants and metazoans and are probably interpreted by these organisms in a slightly different manner (Fischer et al., 2006; Loidl, 2004; Naumann et al., 2005).

4.6 Possible mechanisms of action

Although the mechanism of action of the DET1 complex is still unclear, as suggested above the complex may act by recruiting chromatin-modifying enzymes such as histone methyltransferases. Although the DET1 complex appears to be part of a bona-fide ubiquitin ligase complex in plants (Yanagawa et al., 2004) no substrates have been identified to date. Given that the complex binds chromatin, it is tempting to speculate that it may be involved in chromatin ubiquitination.

Unlike polyubiquitination, monoubiquitination does not appear to target proteins for degradation (Gill, 2004; Hicke, 2001), rather this posttranslational modification acts to regulate the activity of its target (Kaiser et al., 2000). All the core histones are subject to mono-ubiquitination (Hicke, 2001; Wang et al., 2006) although mono-ubiquitination of H2B (H2Bub1) and H2A (H2Aub1) are the most abundant and studied histone monoubiquitination events (Jason et al., 2002). In the yeast *Saccharomyces cerevisiae* monoubiquitination of H2B is catalysed by the Rad6 E2 (Robzyk et al., 2000) and Bre E3 ligase (Hwang et al., 2003) and is important for transcriptional activation. H2Bub1 appears to be necessary to allow for di and tri-methylation of H3K4 (H3K4me2, H3K4me2) by the Set1 histone methyltransferase, which in the absence of H2Bub1 can only catalyze monomethylation (H3K4me1) (Shahbazian et al., 2005). The reason for the requirement for H2Bub1 for Set1 di/trimethylation activity is still not understood, but after H3K4me3 is catalysed by Set1, ubiquitin is cleaved from the H2B by the ubp8 protease which allows full activation of the gene (Daniel et al., 2004).

Unlike H2Bub1 which is associated with gene activation, the H2Aub1 chromatin modification is mainly associated with gene silencing and repression. In mammals H2Aub1 consists of 5-10% of total H2A (Jason et al., 2002), with the largest concentration occurring on the inactive X-chromosome (Smith et al., 2004). Recently it was shown that in metazoans H2A monoubiquitination is catalysed by the PCR1 complex (Polycomb repressor complex 1; (Wang et al., 2004). The PCR1 complex is not conserved in plants and in agreement with this, plants to not appear to contain readily detectable levels of H2Aub (de Napoles et al., 2004).

Recently it was demonstrated that in mammalian cells a complex containing DDB1, DDB2 and Cul4 is required for ubiquitination of H3 and H4 in response to DNA damage (Wang et al., 2006) and re-establishment of mono-ubiquitinated H2A (H2AK119ub1) after repair of DNA damage (Kapetanaki et al., 2006). While the presence of H2Aub1 in plants is still speculative, given the repressive nature of this modification, H2A ubiquitination by a DET1/DDB1/CUL4 containing complex could account for the proposed repressor function of this complex.

4.7 Possible mechanisms of action (non-chromatin)

COP1 is responsible for maintaining several positive regulators of light signalling such as LAF1, HY5 and HYH at low levels in the dark by ubiquitin-mediated proteolysis (Holm et al., 2002; Saijo et al., 2003; Seo et al., 2003). In the dark COP1 is also thought to interact directly with the DET1 complex. Here we have demonstrated that myc-tDET1 binds directly to the CAB2 and HEMA1 promoters, as does the COP1 target HY5 (and presumably HYH) (Chattopadhyay et al., 1998) (Maxwell et al., 2003; McCormac and Terry, 2002). This suggests that DET1 could repress photomorphogenesis by recruiting COP1 to these promoters, where it acts to degrade its targets before they can activate transcription.

The possibility that the DET1 complex is recruited to target promoters by recognition by COP1 of HY5 or other substrates should not be excluded either. COP1 would thus recruit the DET1 complex to such loci, where it would bind directly to the chromatin and hold the complex in place once COP1 has degraded its target. COP1 is nuclear localized only in the dark (von Arnim et al., 1997), so another mechanism would clearly be needed to explain the mechanism of action of the DET1 complex at the POR-A and FT promoters.

Other possible mechanisms of recruitment of the DET1 complex to specific promoters could include targeting by transcription factors acting as negative regulators of transcription, such as PIF3 (Bauer et al., 2004). Another possible mechanism could be targeting by small RNAs (Horn and Peterson, 2006; Jia et al., 2005).

The activation of light induced promoters is likely to be due to a number of complementary and interacting pathways. The cryptochrome photoreceptors are in a complex with COP1, and on light activation they rapidly inhibit itsubiquitin ligase activity (Wang et al., 2001). In response to light COP is also transported out of the nucleus by a slower, unknown mechanism (von Arnim and Deng, 1994). The inhibition and exclusion from the nucleus of COP1 allows the build up of HY5, HYH and other transcription factors that promote the transcription of light induced genes. Light activation of the phytochrome photoreceptors causes them to enter the nucleus were they interact with negative regulators of light signalling such as PIF3 and target them for degradation by ubiquitin mediated proteolysis (Bauer et al., 2004, Park, 2004 #101). The loss of these negative regulators from the promoters of light active genes would probably cause the loss of transcriptional co-repressors (and maybe also the DET1 complex) and at least partially releave the repression on light activated genes. Activated phytochromes also interact with and are thought to activate transcription factors that are positively regulators of light signalling and are thought to help them promote transcription of their target genes through mechanisms which are not yet known but may include helping to target the transcription factor recruit or activate transcriptional co-activators and components of the transcriptional apparatus (Quail, 2002b). The chromatin of transcribed genes is acetylated by histone acetyltransferases that are components of the transcriptional apparatus (Fischle et al., 2003). Acetylation of the histone tails and particularly that of H2B has been shown to abolish the binding of DET1 to chromatin (Benvenuto et al., 2002). So the increase in histone acetylation at these light induced promoters should cause the dissociation of the DET1 complex (and any associate repressive activity) from these promoters, thereby further relieving repression and allowing full activation of the promoter.

It is becoming increasingly obvious that plants modulate their gene expression patterns through a variety of epigenetic mechanisms. For example, DNA-methylation-induced gene silencing is heritable both mitotically and meiotically, ensuring that a particular gene expression pattern is handed down from generation to generation and appears to be principally used to silence repetitive sequences and invasive DNA (Tariq and Paszkowski, 2004). It could also in principle be used to pass on gene expression patterns that confer an advantage in a particular environmental context. Histone modifications such as methylation often act together with DNA methylation but are not generally meiotically heritable and thus they reset at each generation. Some histone methylation is carried out by the Polycomb and Trithorax complexes,

which act to lock in repressed and activated gene expression states, respectively (Cavalli, 2002). In plants the major role of the Polycomb complexes appears to be during development and for establishment of cell fate, which once set is usually maintained throughout the life cycle of the organism (Hsieh et al., 2003; Schubert et al., 2005). Interestingly, Polycomb proteins have been implicated in one developmental transition in plants, vernalisation (Bastow et al., 2004), where they act to maintain the silencing of the *FLC* gene. It is perhaps significant to note that in this example, *FLC*, once silenced, remains so for the life-cycle of the organism.

In this context it is interesting to consider the possible role of the DET1 complex in gene silencing in light of its putative targets identified here, which unlike those of DNA methylation and the Polycomb complex, are only silent at certain developmental stages and later must be activated. This indicates that DET1 complex-mediated silencing must be readily reversible. The DET1 complex appears to co-localize with H3K9me2, which was thought until recently to be a stable epigenetic mark like DNA methylation, but enzymes that demethylate histones have recently been identified (Shi et al., 2004). Histone demethylases are conserved in plants (He et al., 2003), with 25 putative histone de-methylases encoded in the *Arabidopsis* genome, suggesting a mechanism for removing DET1-associated H3K9me2 when DET1-mediated repression is relieved.

The role of the DET1 complex may be to avoid accidental activation of a gene until the activator has reached a certain threshold. This could help to reduce transcriptional noise and to make gene regulation more robust. Such a mechanism could be particularly important in muilticellular organisms such as plants.

4.8 Future Directions

In order to better understand the role of the DET1 complex, a comprehensive survey of its targets should be carried out to determine which gene promoters and other genomic regions that it is binding to. This could be done using the ChIP-chip method where chromatin immunoprecipitation is followed by hybridization of the immunoprecipitated DNA to a DNA microarray (Hanlon and Lieb, 2004). This is done using Tiling DNA microarrays that contain probes corresponding to the entire genome or a genomic region. For Arabidopsis a spotted PCR product array of chromosome 4 has successfully been used for ChIP-chip (Lippman et al., 2004; Martienssen et al., 2005), while Arabidopsis whole genome tiling arrays have also been described using both the Affymetrix platform (Yamada et al., 2003) and the

NimbleGen MAS (Maskless Array Technology) platform (Stolc et al., 2005). ChIP-chip experiments would allow identification of putative targets of the DET1 complex in a semi-unbiased manner. Putative targets could then be confirmed by conventional ChIP followed by semi-quantitative PCR. ChIP experiments should also be repeated using antibodies against, or epitope tagged versions of other components of the DET1 complex (e.g. DDB1, COP10, CUL4) to see if they also localise to the same loci as DET1.

The data presented in this thesis suggest a correlation between myc-tDET1 binding and H3K9me2. In order to understand the mechanism by which DET1 is recruited to and represses its targets, the chromatin modifications (the histone code) at these loci should be investigated. Further ChIP experiments with antibodies against modified histones should be performed to better characterise the chromatin environment at the DET1 loci. Repeating these experiments in the *det1* and other mutant backgrounds will help us to understand if DET1 is using chromatin remodelling or histone modifications to repress its targets.

To investigate whether particular chromatin modifications or histone codes are required to recruit DET1 to its targets, ChIP experiments can be performed in mutants of histone modifying proteins (such as the histone methyltransferases or histone deacetylases) or plants treated with Trichostatin-A (a histone deacetylase inhibitor) to see if myc-tDET binding to its targets is affected.

Once DET1 targets have been identified, literature and publicly available microarray data can be used to predict other components involved in the pathway, either for targeting of the DET1 complex to specific loci or in maintaining repression at the target loci. For example a survey of the literature reveals a number of proteins that bind the *CAB2* promoter (HY5, HYH, CCA1 etc.), while examination of the publicly available microarray data reveals a number of mutants which have mis-regulated expression of *FT* or other DET1 targets (https://www.genevestigator.ethz.ch/). The role of these putative components and other known DET1 associated proteins such as DDB1, DDB2, COP10, COP1 and CUL4 should be investigated by crossing epitope-tagged DET1 into the respective mutant backgrounds and performing ChIP experiments to see if they are required for targeting DET1 to these loci.

In the case of light-regulated loci time course experiments through the dark to light transition (and vice-versa) to determine the kinetics of DET1 recruitment and the

appearance of certain histone modifications may help to elucidate the mechanism of DET1 recruitment and its mechanism of action.

As the components of the DET1 complex have been implicated in DNA damage repair it would also be useful to characterise the behaviour of the DET1 complex in response to UV light and other DNA damaging agents. Possible experiments could include the examination of the recruitment of DET1 to sites of DNA damage using immunofluorescence methods, chromatin association experiments and/or interaction experiments to see if the DET1 complex interacts with other factors in response to DNA damage.

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Appendices

Appendix A: Oligonucleotides

Tags (5'→3')

Fscs: GATCCGGTACAGATTTCGGACATGCGGCCGCATAAGTAGCTGAC
Rscs: TCGAGTCAGCTACTTATGCGGCCGCATGTCCGAAATCTGTACCG
(Annealing primers for generation of *pENTR-stop* vector)

FnSTR: TCGACATGTGGAGCCATCCGCAGTTCGAAAAAGGCGGCAGCGGCG
RnSTR: GATCCGCCGCTGCCGCCTTTTTCGAACTGCGGATGGCTCCACATG
(Annealing primers for generation of N-terminal strep tag entry cassette)

FcSTR: GGCCGCAAGCGGCGGATGGAGCCATCCGCAGTTCGAAAAATAGC
RcSTR: TCGAGCTATTTTTCGAACTGCGGATGGCTCCATCCGCCGCTTGC
(Annealing primers for generation of C-terminal strep tag entry cassette)

Fn3HA: GAAGTCGACATGTCGCGATACCCCTAC
Rn3HA: GAAGGATCCTCCACTGCTAGCGGCGTAG
(PCR primers for generation of N-terminal 3x HA tag epitope entry cassette)

Fn3HA: AGCGGCCGCAAGCGGAGGCCTGTCGCGATAC
Rn3HA: GAACTCGAGAGTACTGCTCTAGGCTTAGTCGGGCAC
(PCR primers for generation of C-terminal 3x HA tag epitope entry cassette)

FnMYC: GAGTCGACGGTATCGATTTAAAGC
RnMYCL: GAAGGATCCCGGGCTTCCGGAATTCAAGTCCTCTTC
(PCR primers for generation of N-terminal 6x MYC epitope tag entry cassette)

FcMYC: GAAGCGGCCGCACAAGCTATGGAGCAAAAGC
RcMYC: GAACTCGAGTCAGGAATTCAAGTCCTC
(PCR primers for generation of C-terminal 6x MYC epitope tag entry cassette)

FnGST: GAAGTCGACATGTCCCCTATACTAGGTTATTG
RnGST: ACGGGATCCACGCGGAACCAGATC
(PCR primers for generation of N-terminal GST tag entry cassette)

FcGST: AGCGGCCGCAAGCGGAGGCATGTCCCCTATACTAGG
RcGST: GAACTCGAGCTAATCCGATTTTGGAGGATGG
(PCR primers for generation of N-terminal GST tag entry cassette)

FnEYFP: GGGTCGACATGGTGAGCAAGGGCG
RnFRET: CCGGATCCAGGCTTGTACAGCTCGTCCATG
(PCR primers for generation of N-terminal ECFP or EYFP tag entry cassette)

FcFRET: GGGCGGCCGCACCTATGGTGAGCAAGGGCG
RcEYFP: CCCTCGAGTTACTTGTACAGCTCGTCCATG
(PCR primers for generation of C-terminal ECFP or EYFP tag entry cassette)

Genes (5'→3')

FnDDB1:   GAA<u>GGATCC</u>ATGAGTGTATGGAACTACG
RnDDB1:   GTAGGCTTTGGGCAACC (*Xho* I)
(PCR primers for amplifying N-terminal fragment of *tDDB1*, *Bam*H I restriction site is underlined)

FcDDB1: GAAGGATCCCTTGTCATGCGACTACC (*Pst* 1)
RcDDB1: AT<u>GCGGCCGC</u>ATGCAACCTTGTCAACTC
(PCR primers for amplifying C-terminal fragment of *tDDB1*, *Not* I restriction site is underlined).

The complete 3500 kb *tDDB1* clone was obtained by ligating the N and C terminal fragments to the central 3000 kb of a sequences *tDDB1* clone using the *Xho* I and *Pst* I sites respectively.

FDDB2: AA<u>GGATCC</u>ATGCGTAGAAGAAGTTTGTTTCC
RDDB2: GAAT<u>GCGGCCGC</u>ACTTCTCCTGGATTTATATGG
(PCR primers for amplifying *tDDB2*, *Bam*H I and *Not* I restriction sites are underlined)

DDB2mF GACAAATGATGG**T**TCCATATATGC
DDB2mR ATATATGGA**A**CCATCATTTGTCGG
(PCR primers removal of an internal *Bam*H I site in *tDDB2* by PCR mutagenesis, bold nucleotides indicate and A→**T** or T→**A** substitution with respect to the native sequence)

FDET: GAA<u>GGATCC</u>ATGTTCAAAACTAACAATGTTACC
RDET: GAAT<u>GCGGCCGC</u>TTATCGACGAAAATGG
(PCR primers for amplifying *tDET1*, *Bam*H I and *Not* I restriction sites are underlined)

FH2A: GAA<u>GGATCC</u>ATGGATGCTACTAAGACAACC
RH2A: GAAT<u>GCGGCCGC</u>TGCCTTCTTCGGTGAC
(PCR primers for amplifying *tH2A*, *Bam*H I and *Not* I restriction sites are underlined)

FH2B: GAA<u>GGATCC</u>ATGGCACCAAAGGCAGG
RH2B: GAAT<u>GCGGCCGC</u>ATTGCTAGTAAACTTGGTG
(PCR primers for amplifying *tH2B*, *Bam*H I and *Not* I restriction sites are underlined)

Fubi: CC<u>GGATCC</u>ATGCAGATATTTGTTAAGACA
RubiST: GT<u>GCGGCCGC</u>TTACCCACCACGTAGACGG
(PCR primers for amplifying (mono) *tUbiqutin*, *Bam*H I and *Not* I restriction sites are underlined)

Sequencing and PCR screening primers (5'→3')

T7: TAATACGACTCACTATAGGG
SP6: ATTTAGGTGACACTATAG
(for sequencing of PCR amplified constructs in the *pCR 2.1* (Topo) vector)

pENTR-5': CTACAAACTCTTCCTGTTAGTTAG
SeqL-B: AACATCAGAGATTTTGAGACAC
(for sequencing and PCR screening of constructs in the entry cassettes (and other *pENTR* (invitrogen) derived vectors)

35sF: GACATCTCCACTGACGTAAGG
35sR: CTCAACACATGAGCGAAACC
(for sequencing and PCR screening of constructs in plant binary vectors containing the 35S promoter and terminator)

Fbar: GCCGACATCCGCCGTGCCAC
Rbar: GTCCAGCTGCCAGAAACCCA
(for PCR screening of plants transformed with a vector conferring resistance to Basta, gives a 479 bp product)

FnptII: GGATTGCACGCAGGTTCTCC
RnptII: AACTCGTCAAGAAGGCGATA
(for PCR screening of plants transformed with a vector conferring resistance to Kanamycin, gives a 772 bp product)

Fhpt: ATGAAAAAGCCTGAACTCAC
Rhpt: GTTTCCACTATCGGCGAGTA
(for PCR screening of plants transformed with a vector conferring resistance to Hygomycin B, gives a 989 bp product)

Appendix B: Constructs

>tDETcoding
GGATCCATGTTCAAAACTAACAATGTTACCGCCAGGCTTTTTGAGCGCCAGATTTGCACCCC
TGCTCCTGGCACCAGCATCCATCGTGCCAGAAGATTTTATGAGAACGTTGTACCAAGTTATA
CCATATACGATGTTGAATGTCCCGACCATTCATTTCGCAAGTTCACGGATGACGGTCTATAT
TTTGTAAGTTTCAGCCGAAACCATCAGGATCTGGTTGTTTATAGACCAACATGGCTGACATT
TTCCTGCAAAGAAGAAGATTGTGATACTCATGATCTTCCTTTGAAAGCTAGAAAGTTTGAGA
GCTTCTTCACACAGTTGTACAGTGTTACTCTTGCTTCTAGTGGGGAACTTATATGCAAAGAT
TTCTTTCTCTATATGGAGAGCAACCAATTTGGACTCTTTGCAACTTCAACTGCACAAATTCA
TGATGCACCTCCTACTGGAGGGGCAATTCAGGGAGTCCCTTCAGTTGAAAAAATAACTTTCC
ACCTTTTGAGGTTGGTGGATGGAGCTATACTTGACGAAAGGGTTTTCCACAATGATTATGTT
AATTTGGCACATAGCATTGGTGCTTTCTTGTATGATGATTTGCTTGCTATAGTGTCTCTTCG
TTATCAAAGAATACACATCCTTCAGATCAGAGATTCTGGAGATCTTGTTGATGTACGAGCAA
TTGGGGAATTCTGCCGTGAAGATGATGAACTTTTTCTCAATTCCAATTCCCAGGTGCTTGTA
AATCATGTTGGAAATGGTTTTCATCATAGTCTGCCTCAATCAGAAACTTCTTTCCTGAGCGG
TATAAAGCAACGGCTGCTTTCATATATATTTCGAGGTATATGGAATGAAGCTGACCAAACCA
TGAGAGTGCAGTGCCTGAAGAAGAAGTTTTACTTCCACTTTCAAGATTACATTGACTTGATT
ATCTGGAAGGTGCAGTTTTTGGACCGACATCACCTGTTGATCAAGTTTGGCAGTGTTGATGG
TGGGGTATCCCGAAATGCTGACATCCATCCTTCTTTTTTTGCTGTTTACAATATGGAGACTA
CTGAAATTGTTGCATTTTATCAGAACTCAGCCGATGAGCTTTATTTCTTGTTCGAGCTGTTC
AGCGACCATTTTCACGTTTCATCCAAAAGTTCATTACATATGAACTTCATGTCCTCACACTC
AAACAACATCCACGCCCTCGAGCAACTAAGGTGTACAAAGAACAAAGCAACCAATTTCTCTC
AATTTGTTAAGAAATGATGGCTTCCTTGCCTTGTAGTTGTCAGTCTCAGAGTCCTTCCCCA
TATTTTGACCAATCTCTCTTCAGGTTTGACGAGAAGCTTATTTCAGCTATTGACCGCCATAG
ACAGTCTACTGACCATCCAATCAAATTCATTTCTAGAAGACAACCCAATATCCTGAAATTCA
AAATGAAGCCAGGACCTGAAGCTGGCAGCACAGATGGGCGAACTAAGAAGATCTGTTCCTTC
CTCTTCCACCCAATATTACCCCTTGCACTTTCTGTTCAACAAACCTTGTTTCTGCAGGCATC
AGTTGTAAATATCCATTTTCGTCGATAAGCGGCCGC

MFKTNNVTAR LFERQICTPA PGTSIHRARR FYENVVPSYT IYDVECPDHS FRKFTDDGLY
FVSFSRNHQD LVVYRPTWLT FSCKEEDCDT HDLPLKARKF ESFFTQLYSV TLASSGELIC
KDFFLYMESN QFGLFATSTA QIHDAPPTGG AIQGVPSVEK ITFHLLRLVD GAILDERVFH

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NDYVNLAHSI GAFLYDDLLA IVSLRYQRIH ILQIRDSGDL VDVRAIGEFC REDDELFLNS
NSQVLVNHVG NGFHHSLPQS ETSFLSGIKQ RLLSYIFRGI WNEADQTMRV QCLKKKFYFH
FQDYIDLIIW KVQFLDRHHL LIKFGSVDGG VSRNADIHPS FFAVYNMETT EIVAFYQNSA
DELYFLFELF SDHFHVSSKS SLHMNFMSSH SNNIHALEQL RCTKNKATNF SQFVKKMMAS
LPCSCQSQSP SPYFDQSLFR FDEKLISAID RHRQSTDHPI KFISRRQPNI LKFKMKPGPE
AGSTDGRTKK ICSFLFHPIL PLALSVQQTL FLQASVVNIH FRR*
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>tDDB1

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GGATCCATGAGTGTATGGAACTACGTGGTTACGGCTCACAAACCAACAAATGTTACACATTC
CTGTGTTGGCAATTTCACCGGTCCTCAAGAGCTCAATCTTATCATTGCGAAATGTACTCGAA
TCGAGATTCATTTACTTACTCCCCAAGGTTTACAGCCTATGTTAGATGTGCCAATATATGGG
AGGATCGCGACACTTGAGCTTTTTCGTCCTCACGGTGAAACACAAGATCTTCTCTTCATCGC
AACAGAGCGATATAAATTCTGTGTCCTTCAATGGGATACTGAGGCATCTGAAGTTATCACAA
GAGCAATGGGAGATGTGTCAGACCGAATAGGCCGTCCCACAGATAATGGTCAGATTGGTATA
ATTGATCCAGATTGCAGATTGATCGGGCTACATCTTTATGATGGACTATTTAAGGTTATTCC
ATTTGATAACAAAGGCCAACTGAAGGAAGCTTTTAACATCAGGCTCGAGGAGCTTCAAGTTT
TAGATATTAAATTCTTGTATGGTTGCCCAAAGCCTACAATTGTTGTTCTATATCAGGATAAC
AAGGATGCCCGGCATGTCAAAACATATGAGGTGTCCTTGAAAGACAAAGATTTTATTGAAGG
GCCATGGGCTCAAAATAATCTTGATAATGGAGCTTCTTTGCTAATACCAGTACCTCCACCAC
TGTGTGGTGTATTGATTATTGGAGAAGAAACCATCGTTTATTGCAGCGCTTCAGCTTTTAAG
GCTATCCCAATTAGACCTTCTATCACAAGAGCATATGGGCGGGTTGATGCTGATGGTTCTCG
ATATTTGCTTGGGGATCATAATGGGCTTCTTCACCTACTTGTAATCACTCATGAGAAGGAGA
AAGTTACCGGACTCAAAATTGAGCTACTGGGGGAAACTTCTATTGCATCAACCATATCATAC
CTAGACAATGCTTTTGTCTTCATTGGCTCAAGCTACGGAGATTCACAGCTTGTAAAGCTCAA
TCTCCAGCCTGACACCAAAGGTTCTTATGTGGAAGTTCTAGAGAGATATGTCAATTTAGGAC
CTATTGTGGACTTCTGTGTTGTTGATCTGGAAAGGCAAGGTCAAGGTCAGGTTGTAACTTGC
TCTGGAGCCTATAAGGATGGATCACTTCGTATTGTTCGAAATGGAATTGGCATAAATGAACA
GGCGTCTGTGGAACTACAAGGGATCAAAGGAATGTGGTCTCTTAGATCTGCTACTGATGATC
CATATGACACATTCTTGGTTGTTAGCTTCATTAGTGAGACACGCGTTTTGGCTATGAACCTT
GAGGATGAGCTGGAAGAAACTGAGATAGAAGGCTTCAATTCTCAAGTCCAGACCTTGTTTTG
TCATGATGCTGTATACAACCAGCTTGTTCAGGTTACTTCAAATTCTGTTAGATTGGTCAGTT
CTACCTCTAGAGATCTGAAAAACGAGTGGTTTGCCCCAGTCGGCTACTCGGTCAATGTTGCA
ACTGCTAATGCCACTCAGGTACTATTGGCTACTGGGGGTGGCCATCTGGTATACCTAGAAAT
TGGTGATGGGGTGTTGAATGAAGTAAAATATGCCAAGTTGGATTATGATATCTCGTGCCTGG
ACATAAATCCAATTGGTGAAAATCCGAACTACAGTAACATTGCAGCAGTTGGAATGTGGACA
GACATAAGTGTCAGGATATATTCACTTCCTGACTTGAATCTCATTACAAAGGAACAGCTAGG
AGGGGAGATAATTCCTCGTTCTGTTCTGATGTGTTCCTTCGAAGGGATATCTTATCTACTAT
GTGCTTTGGGAGATGGCCATCTCTTGAATTTTGTATTGAGCATGAGTACTGGTGAGCTGACA
GATAGGAAAAAAGTTTCTCTTGGGACACAGCCCATAACACTTCGTACATTCTCATCTAAAGA
TACTACACATGTCTTTGCTGCCTCCGATAGGCCAACAGTTATTTACAGCAGTAACAAGAAGC
TGCTTTATAGCAATGTAAATCTAAAAGAAGTTAGTCATATGTGCCCATTCAATGTTGCAGCT
TTTCCAGACAGCCTTGCAATCGCTAAAGAAGGTGAGTTAACAATTGGCACTATTGATGAAAT
TCAAAAGCTTCACATTCGTTCAATACCCCTTGGGGAGCATGCACGTCGCATCAGCCATCAAG
AGCAGACCCGGACATTTGCTCTATGCAGTGTGAAGTATACTCAGTCAAATGCAGATGATCCT
GAAATGCATTTTGTCCGCCTGTTGGATGATCAGACATTTGAGTTCATATCAACATATCCCCT
TGACCAATTTGAATATGGCTGTTCCATACTAAGCTGCTCCTTTTCTGATGATAGTAATGTGT
ATTATTGCATTGGAACTGCATATGTGATGCCAGAGGAAAATGAACCTACTAAGGGCCGAATT
TTAGTTTTTATAGTTGAAGATGGAAAGCTCCAGCTAATTGCTGAGAAGGAAACTAAGGGAGC
TGTCTACTCTCTAAATGCCTTCAATGGGAAACTGCTTGCTGCAATCAATCAGAAGATTCAAT
TGTACAAGTGGGCTTCGCGTGAGGATGGTGGCAGCCGAGAATTGCAGACAGAATGTGGACAC
CATGGTCATATATTAGCTCTTTATGTTCAAACACGTGGGGATTTCATTGTTGTTGGTGATTT
GATGAAATCCATTTCTCTGCTGATTTTCAAGCATGAAGAGGGTGCTATAGAGGAGCGAGCCA
GAGACTATAATGCAAATTGGATGTCAGCTGTTGAGATTCTCGATGATGACATTTATCTTGGT
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>tDDB2
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>tH2A

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>tH2B

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>tUBlst

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