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Ubiquitin-ligase-mediated transcription initiation in cellular stress defences

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Abstract

Accurate regulation of gene transcription is essential for organismal survival, and is orchestrated by myriad transcription factors and cofactors (TFs). Little is known about how the intrinsic activity of TFs is controlled. Recent work has indicated that the selective proteolysis of TFs provided by the ubiquitin-proteasome system (UPS) plays an important role in stimulating gene expression through a 'destruction-activation' mechanism, whereby the degradation of a 'used' TF is thought to stimulate further 'fresh' TF binding and reinitiate gene transcription. TFs are targeted to the proteasome via E3 ligases that mediate the addition of ubiquitin molecules to form a chain on the substrate TF. These polyubiquitin chains may be extended by E4 ligases, which recognize substrates with four or more ubiquitin molecules, amplifying substrate targeting to the proteasome.

In plants the immune response to many pathogens is regulated by the hormone salicylic acid (SA), which operates through the transcriptional coactivator NPR1 to induce large scale changes in gene expression. Proteasome-mediated degradation of NPR1 appears to be required for the activation of its target genes. Mutation of the E3 ligase prevents ubiquitination of NPR1, leading its to stabilisation and suppression of transcription. Chapter 3 of this work identifies the first E4 ligase, UBE4, involved in NPR1 regulation. Mutation of UBE4 resulted in reduced capacity to polyubiquitinate substrates and stabilized NPR1. In contrast to E3 ligase mutants, however, mutant *ube4* plants displayed increased NPR1 target gene expression. These results suggest that initial ubiquitination of NPR1 may stimulate its ability to initiate transcription and that subsequent ubiquitin chain elongation limits NPR1 activity by targeting it to the proteasome.

Chapter 4 describes a ubiquitin-protein-ligase (UPL) which is both novel and crucial to the SA-mediated defence response. Mutation of this UPL leads a large reduction in total

cellular polyubiquitinated proteins and was associated with strongly enhanced disease susceptibility. Gene expression profiling of *upl* mutants revealed an intimate connection between cellular polyubiquitination and appropriate activation of SA-responsive gene expression programmes.

Destruction activation was first described in yeast and is required for the regulation of yeast amino acid synthesis TF GCN4. GCN4 requires proteasome-mediated degradation to induce genes involved in amino acid production. Chapter 5 investigates the role of two E4 ligases in GCN4 turnover. While one mutation had little effect of GCN4-mediated transcription a second increased basal transcriptional levels, suggesting that an E4 is required for the prevention of spurious GCN4-mediated transcription.

In summary the work presented here describes cellular mechanisms by which global and substrate-specific polyubiqutination are vital to regulation of gene transcription.

Lay summary

Living organisms must respond quickly and accurately to many developmental and environmental cues in order to survive. To produce the right response to a specific cue, the correct genes must be expressed at the appropriate time and level. Control over gene expression is provided by specific proteins, called transcription factors (TFs), but what regulates TF activity is poorly understood. One mechanism of controlling TFs is through their regulated destruction. In this process the TFs are repeatedly tagged with a molecule called ubiquitin by enzymes known as ubiquitin-ligases. Ubiquitin tagging acts as a signal for TF destruction by the proteasome, which functions as the cell's trash-can that shreds unwanted proteins and recycles their building blocks. This work investigates how aspects of the tagging process of TFs by ubiquitin ligases alter the activity of TFs. Results indicate that the ubiquitin-ligases which act on a single TF or on total cellular ubiquitination levels can influence specific gene expression programmes, thereby regulating an organism's response to internal or external stresses.

Declaration

I declare that the work presented here is my own work, unless explicitly stated and that it has not been submitted for a degree or a professional qualification at the University of Edinburgh or any other institution.

James J. Furniss

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26S	26S proteasome
AAA	ATPases Associated with diverse cellular Activities
ACIF1	Avr9/Cf-9 induced F-box 1
ACT	Aspartate kinase, Chorismate mutase, TyrA domain
ACT1	Actin 1
ADH5	Alcohol Dehydrogenase 5
ANAC	Arabidopsis NAC-containing protein
ARG1	Arginine requiring 1
Arm	Armadillo domain
as-1	Activation Sequence 1
ASN1	Asparagine requiring 1
At	*Arabidopsis thaliana*
ATP	Adenosine Tri-Phosphate
AZI1	Azelaic acid Induced 1
BAH	Benzoic Acid Hypersenstive 1 - dominant
BAK1	BRI1-associated receptor kinase 1
bHLH	basic Helix-Loop-Helix
BIK1	Botrytis Induced Kinase 1
BRCA2A	Breast Cancer 2-like 2A
BTB	Bric-à-brac, Tramtrack and Broad complex
C-	Carboxyl
CBP	CREB-binding protein
CBP60	Calmodulin-Binding protein-like 60
CDC4	Cell Division Cycle 4
CDC48	Cell Division Cycle 48
CHX	Cycloheximide
CP	20S Core Protease
CPA2	Carbamyl phosphate synthetase A 2
CPR1	Constitutive expresser of PR genes 1
CRL	Cullin Ring Ligase
CUL3	Cullin 3
DIR1	Defective in Induced Resistance 1
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribo-Nucleic Acid
DUB	Deubiquitinase
DTT	Dithiothreitol
EDS5	Enhanced Disease Susceptibility 5
EDTA	Ethylene-Diamine-Tetra-Acetic acid
EGL3	Enhancer of GL3
EIL1	Ethylene Insensitive 3-Like
EIN3	Ethylene Insensitive 3
ERα	Estrogen Receptor α

Fbw7	F-box and WD40 domain containing protein 7
FPKM	Fragments Per Kilobase per million Mapped reads
FLS2	Flagellin Sensitive 2
G3P	Glyercol-3-Phosphate
GAAC	General Amino Acid Control
GAL4	Galactose metabolism 4
GCN4	General Control Non-inducible 4
GFP	Green Fluorescent Protein
GL3	Glabrous 3
GST	Glutathione-S-Transferase
HECT	Homologous to E6-AP Carboxyl Terminus
HIS4	Histidine requiring 4
HR	Hypersensitive Response
HUD	Hormone Up at Dawn
HUL5	HECT Ubiquitin Ligase 5
IB	Immuno-blot
IC	Initiation Complex
ICS1	Isochorismate Synthase 1
IP	Immuno-precipitation
IQ	Isoleucine-glutamine motif
JA	Jasmonic Acid
K48	Lysine 48
Lys	Lysine
MATE	Multi-drug And Toxin Extrusion
MDM2	Mouse Double-Minute 2
MeSA	Methyl-Salicylic Acid
MES	2-(*N*-morpholino)ethanesulfonic acid
MET30	Methionine requiring 30
MS	Murashige and Skoog
MG132	Z-Leu-Leu-Leu-al
N-	Amino
NEM	N-ethylmaleimide
NIMIN	NIM1-Interacting 1
NLR	Nucleotide-binding/Leucine-rich Repeat
NPR1	Non-expressor of PR genes 1
NPR3	Non-expressor of PR genes 3
NPR4	Non-expressor of PR genes 4
NTD	N-terminal domain
Os	*Oryza sativa* (Rice)
PAL	Phenylalanine Lyase
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PD	Pull-Down
PHO85	Phosphate metabolism 85

PHYRE2	Protein Homology/analog Recoginition Engine 2
PMSF	Phenylmethanesulfonyl flouride
PR	Pathogeneisis Related
Pru	Pleckstrin-like receptor
Psm	*Pseudomonas syringae* pv. *maculicola*
PUB	Plant U-Box
qPCR	Quantitative real-time PCR
RAD23	Radiation sensitive 23
RAD51D	Radiation sensitive 51 D
RIN	RPM-interacting protein
RING	Really Interesting New Gene
RNA	Ribo-Nucleic Acid
RNAP	RNA polymerase
RNA-seq	RNA-sequencing
RP	19S Regulatory Particle
RPM1	Resistance to *P. Syringae* pv. *Maculicola* 1
RPN	Regulatory Particle Non-ATPase
RPS2	Resistant to *P. Syringae2*
RPT	Regulatory Particle AAA-ATPase
R-SMAD	Receptor-activated SMAD
RT	Room Temperature
RT-PCR	Reverse Transcriptase PCR
SA	Salicylic Acid
SABP2	Salicylic Acid-Binding Protein 2
SAG	SA *O-β*-glucoside
SAGT	SA glucosyltransferses
SAIL	Syngenta *Arabidopsis* Insertion Library
SALK	The Salk institute
SAR	Systemic Acquired Resistance
SARD1	SAR Deficient 1
SCF	Skp, Cullin, F-box
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis
SE	Standard Error
SID2	SA induction deficient 2 (a.k.a. ICS1)
SMAD	Mothers Against Decapentaplegic homolog
SMC	Structural Maintenance of Chromosome
SMM	Synthetic Minimal Media
SNC1	Suppressor of *npr1-1*, Constitutive
SNI1	Suppressor of *npr1*-1, Inducible
SRb10	Suppressor of SNf1 10
SRC-3	Steroid Receptor Coactivator-3
SSN2	Suppressor of *sni-1*, Inducible
SUMO	Small Ubiquitin-like Modifier

TA	Transcriptional Activator
T-DNA	Transfer-DNA
TF	Transcription Factor
TGA	TGACG sequence specific binding protein
TGA3	TGA1A-related gene 3
TLCK	N-*p*-Tosyl-L-Lysine Chloromethyl Ketone
TMV	Tobacco Mosaic Virus
TPCK	N-*p*-Tosyl-L-phenylalaninie Chloromethyl Ketone
TUBE	Tandem Ubiquitin Binding Entities
UBE4	Ubiquitin ligase E4
UBP6	Ubiquitin-specific Protease 6
UBQ	Ubiquitin
UBR1	Ubiquitin protein ligase component n-Recognin 1
UFD2	Ubiquitin Fusion Degradation 2
UFD4	Ubiquitin Fusion Degradation 4
UIM	Ubiquitin Interaction Motif
UPL	Ubiquitin Protein Ligase
USP14	Ubiquitin Specific Peptidase
WRKY	Tryptophan-Arginine-Lysine-Tyrosine domain containing protein
WT	Wild type
YFP	Yellow Fluorescent Protein

Chapter 1: Introduction

1.1. The ubiquitin-proteasome system

1.1.1. The ubiquitination cascade

Accurate control of gene transcription is crucial to the viability of any organism: failure to regulate gene expression correctly can lead to disease and death. In eukaryotes the rate of gene transcription is highly dynamic and plays a key role in coordinating cellular development and responses to environmental signals and stresses. Cellular responsibility for precise gene regulation primarily falls upon an interlaced network of transcription factors (TFs) and cofactors. However, we know relatively little about the systems that regulate the activities of TFs and therefore the rate and dynamics of gene transcription. Recent research in this field indicates that the Ubiquitin-Proteasome System (UPS) plays an important role in controlling the activities of TFs (Lipford et al., 2005, Spoel et al., 2009, Geng et al., 2012, Vierstra, 2009, Muratani and Tansey, 2003).

In eukaryotic cells, post-translational modification by a single or polymeric chain of ubiquitin modulates protein function and stability (Komander and Rape, 2012). Ubiquitin is a highly conserved, small protein (8.5 kDa) that is covalently attached to a target substrate in a multistep enzymatic pathway. First, a ubiquitin-activating E1 enzyme forms a high-energy thioester linkage to a ubiquitin moiety, which is then passed onto an active-site cysteine residue of a ubiquitin-conjugating E2 enzyme. The E2 enzyme works in physical partnership with an E3 ligase to attach ubiquitin onto a specific lysine (Lys) ε-amino group within the target substrate (Smalle and Vierstra, 2004, Komander and Rape, 2012).

E3s interact with target proteins due to the recognition of degradation motifs known as degrons. Within TFs a degron can overlap with the transcriptional activation

domain, which is the area that interacts with the general transcriptional machinery (Salghetti et al., 2000, Muratani and Tansey, 2003), creating a direct link between transcription and the UPS. Degrons can be revealed through post-translational modifications (e.g. phosphorylation) so substrates can signal for their degradation when the correct conditions are met (Ravid and Hochstrasser, 2008).

Compared to many other eukaryotes, plant genomes encode for disproportionally large numbers of E3 ligases; for example, the *Arabidopsis* genome contains over 1400 different predicted E3 ligase components (Vierstra, 2009), suggesting that protein ubiquitination plays critical roles in plant biology. E3 ligases selectively recruit substrates for ubiquitination and thus provide an important level of specificity to the ubiquitination machinery. Categorization into different classes of E3 is based on the presence of a really interesting new gene (RING), U-box, or homologous to E6-AP carboxyl terminus (HECT) domain, providing distinct ways of binding a partner E2 conjugating enzyme. In addition to single polypeptide E3 ligases, the modular multi-subunit family of Cullin-RING ligases (CRLs) plays prominent roles in protein ubiquitination. The Cullin subunit of CRLs acts as a scaffold to bring together the RING domain-containing protein and a variable adaptor that recruits the target protein (Sadanandom et al., 2012, Vierstra, 2009, Santner and Estelle, 2009).

Conjugation of ubiquitin continues for several rounds as a chain containing the minimum of four ubiquitin molecules is required to signal destruction, however, the target protein may be passed off to an E4 ligase which continues polyubiquitination (Figure 1.1) (Crosas et al., 2006, Saeki et al., 2004, Koegl et al., 1999). Unlike E3 enzymes, E4 ligases do not recognise a specific substrate for ubiquitination; rather they target only the polyubiquitin chain and facilitate extension (Tu et al., 2007, Saeki et al., 2004). Chain cleavage can be performed by the proteasome lid protein regulatory particle non-ATPase

11 (RPN11), which removes the ubiquitin before the target is degraded (Verma et al., 2002), allowing recycling of ubiquitin molecules.

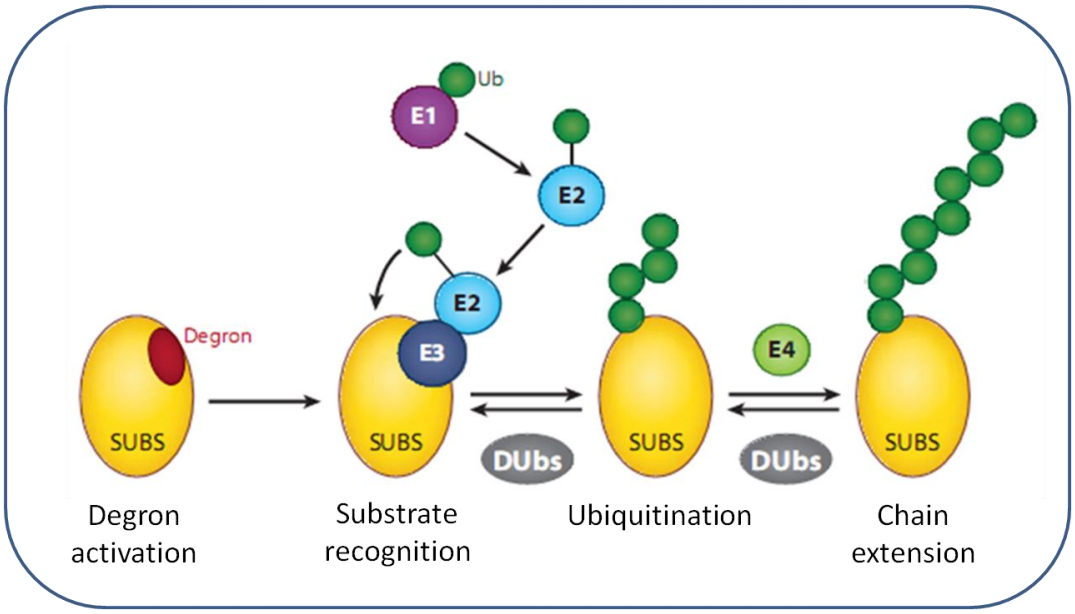

Figure 1.1: The ubiquitination pathway.

A substrate protein (SUBS) destined for the UPS displays a degradation signal (Degron), which is recognised by an E3 ligase. Ubiquitination is achieved by ubiquitin (Ub) being activated by an E1 before being passed to an E2 ubiquitin conjugating enzyme, the E3 mediates initial ubiquitination of the substrate. E4 ligases then extend the ubiquitin chain further. Ubiquitination can be reversed by deubiquitinases (DUbs). Modified from Geng et al. (2012).

Ubiquitin contains seven lysine residues, but for "classical" proteasome-mediated destruction of a target substrate the ubiquitin chain is connected only through residue Lysine 48 (K48). Nonetheless, the remaining lysine residues can support polyubiquitination and chains can also be formed through the N-terminal methionine bonding to the C-terminal glycine. These "non-classical" chains have been shown to have roles in DNA repair, cell cycle progression, innate immunity and inflammation (Ikeda et al., 2010). As E4 ligases

exhibit only limited preference for ubiquitin chain linkage type (Koegl et al., 1999, Saeki et al., 2004, Crosas et al., 2006, Hwang et al., 2010), it is possible that E4 ligases have as yet undescribed non-proteasomal associated roles.

1.1.2. The proteasome

The 26S proteasome is a barrel-shaped, ATP-dependent chambered protease complex containing over 30 distinct subunits with a total size of ≈ 2.5 MDa (Figure 1.2). It is composed of two functionally distinct and individually stable sub-complexes: the 20S core protease (CP) and the 19S regulatory particle (RP). Protein degradation occurs within the CP which comprises of two rings of seven distinct α subunits and two rings of seven distinct β subunits in a symmetrical arrangement ($\alpha_{1-7}\beta_{1-7}\beta_{1-7}\ \alpha_{1-7}$) (Groll et al., 1997). The α rings form an antechamber and although their true role is unknown, they may control the passage of substrates into the anti-chambers created between the α and β rings. This is thought to hold substrates in a 'ready-to-digest' state, so they can rapidly enter the β chambers where they are destroyed by a very high concentration of peptidases (Pickart and Cohen, 2004).

To prevent unwanted degradation of native proteins the CP pore leading to the proteases is gated by the N-termini of the α subunits; even when open the pore is no more than 2 nm across, preventing folded proteins from entering (Groll et al., 2000). In order for ubiquitinated substrates to be degraded they must be recognised and unfolded by the 19S regulatory particle, which can bind to the α rings at both ends of the CP. The RP can be broken down into two further segments of the lid and the base, which are connected to each other via a hinge mechanism provided by the RPN10 subunit (Pickart and Cohen, 2004).

The lid provides target substrate recognition via the ubiquitin interaction motif (UIM) of RPN10 (Lander et al., 2012) and through the pleckstrin-like receptor for ubiquitin (Pru) domain of RPN13 (Schreiner et al., 2008, Husnjak et al., 2008). Deubiquitination of substrates can be performed by the lid subunit RPN11 (Verma et al., 2002). Other lid subunits such as RPN5 and RPN12 may provide additional substrate recognition and scaffold sites (Book et al., 2009, Smalle et al., 2002). Once a substrate has been detected, bound and deubiquitinated, it is passed from the lid to the 19S base sub-complex, of which the main function appears to be the unfolding of substrate proteins and feeding them through the pore in the α-rings. This process is driven by the ATP-dependent unfoldase activities of the Regulatory Particle AAA-ATPase 1-6 (RPT1-6) base subunits (Lander et al., 2012). One of the base's two non-ATPase proteins RPN1 may act as a docking site for the proteasome shuttle protein radiation sensitive 23 (RAD23) which can bind ubiquitinated substrates and hand them to the proteasome, facilitating orderly substrate degradation (Kim et al., 2004, Farmer et al., 2010).

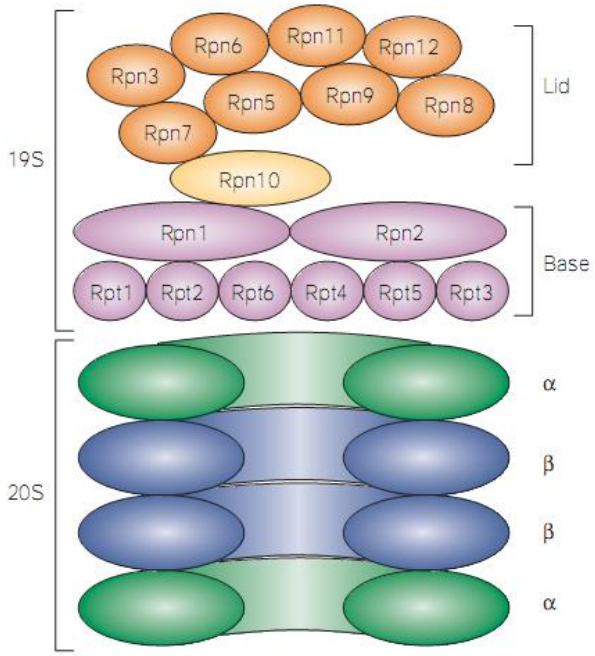

Figure 1.2: Structure of the 26S proteasome.

The proteasome consists of two main sub-complexes; the 20S core particle and the 19S regulatory particle. The 20S is a chambered protease formed from two rings each of α and β subunits and rapidly degrades polypeptides fed into it by the 19S. The 19S can be divided into the lid and base sections. The lid recognises, binds, and deubiquitinates target substrates. The base unfolds substrates and passes them to the 20S in an ATP dependent manner. The 26S and the 19S may also have non-proteolytic roles within the cell. RPN (Regulatory Particle Non-ATPase), RPT (Regulatory Particle AAA-ATPase). Image reproduced from Pickart and Cohen, 2004.

1.2. Salicylic acid and its role in systemic acquired resistance

Successful plant immune responses depend on the rapid recognition of the invading pathogen and subsequent local and systemic transmission of signals that induce resistance throughout all plant tissues. Pattern recognition receptors that recognize conserved pathogen-associated molecular patterns represent the first line of defense, leading to pattern-triggered immunity (Macho and Zipfel, 2014). To subvert immune responses, adapted pathogens have evolved an arsenal of effector proteins that suppress pattern-triggered immunity. The presence of these effector proteins can be sensed by intracellular nucleotide-binding/leucine-rich repeat domain containing (NLR) immune receptors, resulting in effector-triggered immunity (van Ooijen et al., 2007, Jones and Dangl, 2006). Effector-triggered immunity is characterized by rapid onset of programmed cell death (PCD) at the site of infection, which is thought to isolate the invading pathogen. Following pathogen recognition, development of pattern- and effector-triggered immunity requires the immune signaling hormone salicylic acid (SA). Failure to accumulate SA upon pathogen attack results in severe disease susceptibility and inability to launch NLR receptor-mediated PCD (Rairdan and Delaney, 2002, Delaney et al., 1994). Additionally, SA accumulates in tissues adjacent and distant to the site of infection where it induces systemic acquired resistance (SAR), a long-lasting immune response effective against a broad -spectrum of pathogens (Fu and Dong, 2013, Spoel and Dong, 2012).

SA is produced in *Arabidopsis* via two pathways: it is either produced from cinnamate by phenylalanine lyase (PAL) or through conversion of chorismate into isochorismate by isochorismate synthase (ICS1) after which a pyruvate lyase is responsible for the final SA generation step (Chen et al., 2009b). The pathway mediated by ICS1 is the principle pathway in plant immune responses, as *ics1* mutants are only able to generate

approximately 10 % of wild type SA levels after pathogen infection and are highly susceptible to disease (Chen et al., 2009b). Generation of SA is dynamic in response to pathogen perception with transcriptional up-regulation of *ICS1* mediated by the TFs SAR-deficient 1 (SARD1) and its homolog calmodulin-binding protein 60 (CBP60) (Zhang et al., 2010). Repression of *ICS1* is achieved via genes involved in Jasmonic acid (JA) and ethylene signalling (Arabidopsis NAC-containing protein 19 (ANAC019), ANAC055, ANAC072, ethylene insensitive 3 (EIN3) and ethylene-insensitive 3-like 1 (EIL1)) (Bu et al., 2008, Chen et al., 2009a, Yoo et al., 2009, Zheng et al., 2012), indicating SA generation is a focal point for crosstalk between antagonistic signalling molecules.

SA synthesis is likely to be chloroplast localised as enhanced disease susceptibility 5 (EDS5), a multi-drug and toxin extrusion (MATE) transporter, is also involved in SA synthesis and predicted to localize to the chloroplast. Moreover, ICS1 has a plastid transit peptide on its N-terminus suggesting that SA is made in the chloroplast and transported to the cytosol for modification for storage or signalling (Nawrath et al., 2002, Ishihara et al., 2008). At high concentration SA is toxic to plants. To prevent this, SA can be conjugated to other molecules to provide derivatives such as SA *O*-*β*-glucoside (SAG) and volatile methyl-SA (MeSA) (Park et al., 2007). SAGs are formed by the action of SA glucosyltransferses (SAGT) and likely provide a means of storing SA, as SA can be rapidly released via hydrolysation (Dean et al., 2005).

Salicylic acid was first implicated in disease resistance when it was shown to reduce the lesion size caused by tobacco mosaic virus (TMV) infection (White, 1979). SA is essential for SAR because plants carrying the NahG transgene, a bacterial salicylate hydroxylase, are unable to produce SAR (Gaffney et al., 1993, Delaney et al., 1994). Although required for SAR, SA is not the mobile signal within plants, as grafting experiments using rootstocks

unable to accumulate SA were still able to generate disease resistance in distal leaves (Vernooij et al., 1994). More recently MeSA was implicated as the bioactive signalling form of SA (Park et al., 2007). MeSA is generated via methyl-transferase activity on SA: in tobacco MeSA was shown to be required to activate SAR as silencing of the methyl-transferase activity prevented full SAR induction. Furthermore, in infected leaves the SA-binding protein 2 (SABP2), which has methyl-hydrolase activity, was shown to be inhibited by high levels of endogenous SA after pathogen infection, indicating a build-up of MeSA to promote defence activation. In systemic tissue SABP2 is then required to return MeSA into SA, in order to generate SAR (Park et al., 2007). In *Arabidopsis* knockout of methyl-transferase did not prevent SAR, even though MeSA did not accumulate. Additionally most MeSA produced in SAR is lost to volatile emission, suggesting further signals are required within *Arabidopsis* (Attaran et al., 2009). Recently two further molecules have been implicated in the establishment of SAR; the lipid azelaic acid and glyercol-3-phosphate (G3P). Azelaic acid induces the expression of the lipid transfer protein, azelaic acid induced 1 (AZI1), which has been suggested to be involved in the movement of an immune signal. Accordingly, application of azelaic acid leads to the production of immune response gene expression (Jung et al., 2009). G3P is an organo-phosphate produced by reduction of dihydroxoacetone phosphate by G3P-dehydrogenases and the phosphorylation of glycerol via a glycerol kinase. While mutation of these enzymes prevents SAR, it can be reinstated by exogenous application of G3P. It was subsequently discovered that G3P requires the lipid transfer protein, defective in induced resistance 1 (DIR1), in order to move into distal tissues via the symplast and promote SAR (Chanda et al., 2011). Therefore a complex of G3P and DIR1 could be the mobile signal for SAR in *Arabidopsis* (Chanda et al., 2011), but this does not exclude the possibility of other signal molecules being used for SAR activation on a plant and pathogen species-dependent basis. Regardless of the exact mechanism of

transduction the major function of SA is to initiate reprogramming of the transcriptome in challenged tissues to prioritize immune responses over other cellular functions. Accordingly, SA fine-tunes the activity of a network of SA-responsive transcriptional regulators, the concerted action of which establishes disease resistance (Moore et al., 2011).

1.3. NPR1, the master regulator of SA-dependent gene expression

Genetic screens for SA-insensitive *Arabidopsis* mutants have repeatedly identified *npr1* (*non-expresser of PR genes*) mutant alleles (Cao et al., 1994, Delaney et al., 1995, Shah et al., 1997, Glazebrook et al., 1996). *NPR1* encodes a transcription coactivator that in resting cells forms a high molecular weight oligomer in the cytoplasm through intermolecular disulfide bonds between conserved cysteine residues, preventing it from entering the nucleus. Pathogen-induced SA accumulation triggers transient cellular redox changes, resulting in reduction of these disulfide bonds and release of NPR1 monomers (Mou et al., 2003, Tada et al., 2008). NPR1 monomer translocates to the nucleus where it controls the expression of over 2,200 genes in *Arabidopsis* (Kinkema et al., 2000, Wang et al., 2006). The transcriptional cascade orchestrated via NPR1 includes the involvement of several families of TFs. The WRKY family of TFs contains a variety of both positive and negative regulators of NPR1-mediated gene transcription (Pandey and Somssich, 2009, Eulgem and Somssich, 2007, Wang et al., 2005). WRKY transcription factors bind the W-box motif (T)(T)TGAC(C/T), which is over-represented in many NPR1 target genes (Maleck et al., 2000). WRKY regulation is complicated by their overlapping functions and mutual influence on each other (Pandey and Somssich, 2009, Eulgem and Somssich, 2007). There are several WRKY genes playing key roles in the mediation of NPR1-dependent transcription: WRKY18

enhanced the amplitude of expression of 451 NPR1-dependent genes and its knockout increased disease susceptibility. Conversely, WRKY70 and its homolog WRKY54 act as repressors of SA biosynthesis. Because NPR1 directly stimulates expression of many WRKY genes a feedback loop has been proposed that shuts down SA biosynthesis after pathogen challenge has passed (Wang et al., 2006). WRKY70 has also been discovered to act as a direct repressor of NPR1. The application of SA leads to phosphorylation of Serine 55 / Serine 59 and sumoylation of NPR1, thereby dissociating it from WRKY70. Sumolyated NPR1 is then phosphorylated at Serine 11 / Serine 15, which forms a signal amplification loop that triggers the formation of more activated NPR1. Activated NPR1 subsequently binds to the transcription factor TGA1A-related gene 3 (TGA3) to promote target gene expression (Saleh et al., 2015). WRKY58 may also act as an NPR1 repressor; *wrky58* plants are resistant to disease at low levels of SA, suggesting it is required to prevent spurious gene activation at sub-optimal SA levels (Wang et al., 2006).

NPR1 has no intrinsic DNA binding ability and requires additional cofactors to promote transcription. In *Arabidopsis* there are ten TGA TFs, seven of which bind to NPR1. TGA2, TGA3, TGA5, TGA6, TGA7 bind constitutively to activation sequence 1 (*as-1)* regions in the promoters of pathogenesis related *(PR*) genes, however binding affinity in TGA2 and TGA3 is enhanced by the presence of NPR1 (Johnson et al., 2003, Zhou et al., 2000). TGA1 and TGA4 only interact with NPR1 after SA addition. These TGA factors contain two unique cysteine residues that were shown to respond to changes in redox status that accompanies SA signalling. Consequently, SA-induced cellular reduction reduces NPR1 from an oligomeric to monomeric state, while also reducing TGA1 and TGA4, which allows these TGA factors to interact with NPR1 (Lindermayr et al., 2010, Després et al., 2000, Despres et al., 2003). However, TGA1 and TGA4 were shown to have little effect on SA- or pathogen-induced *PR* gene expression, but are mainly involved in basal defences (Kesarwani et al., 2007). In fact

many of the TGA factors have redundant or overlapping roles, making it difficult to tease apart exactly how TGAs and NPR1 interact (Kesarwani et al., 2007, Zhang et al., 2003b, Boyle et al., 2009).

Three NIM1-Interacting (NIMIN) cofactors were also shown to physically interact with NPR1. NIMIN proteins attenuate NPR1-mediated gene expression as over-expression and knockout of these genes repressed and enhanced SAR, respectively (Weigel et al., 2005, Weigel et al., 2001). NIMIN1 probably achieves this by interrupting the TGA-NPR1 transcription complex (Weigel et al., 2005). Presence of EAR repression motifs in NIMIN cofactors has led to the suggestion that SA can interrupt NIMIN binding to the TGA-NPR1 complex (Maier et al., 2011).

To discover repressors of NPR1 a suppressor screen on *npr1-1* mutants was performed and revealed *suppressor of npr1*-1, Inducible (SNI1) as being able to generate defence expression in absence of NPR1 (Li et al., 1999). In a bid to discover how SNI1 functioned a screen for suppressors of SNI1 revealed radiation sensitive 51D (RAD51D), breast cancer 2-like 2A (BRCA2A) and *suppressor of sni-1,* 2 (SSN2) (Song et al., 2011, Wang et al., 2010, Durrant et al., 2007), which are all involved in DNA recombination and repair. SNI1 was subsequently revealed to be a factor in the structural maintenance of chromosome (SMC) 5/6, required for the resolution of DNA intermediates. Mutation of *sni1* produced high basal levels of *PR* genes, heightened sensitivity to SA, and apparently contradictory increased homologous recombination alongside heightened levels of DNA damage. Yan et al. (2013) hypothesize this is due to homologous recombination becoming "stuck" in the final stages in *sni1* mutants. Compensation of the sni1 phenotype in Suppressors of SNI1 are thought to completely block homologous recombination and force DNA repair via the non-homologous end joining pathway instead (Yan et al., 2013).

Therefore SNI1 may play an important role in balancing short-term defence responses with long-term genome integrity (Yan et al., 2013, Durrant et al., 2007).

1.4. Ubiquitin-mediated regulation of transcription

1.4.1. Ubiquitin-mediated suppression of SA-responsive gene transcription

NPR1 protein contains an N-terminal BTB (Bric-à-brac, Tramtrack and Broad complex) domain and a C-terminal ankyrin repeat domain (Cao et al., 1997, Ryals et al., 1997, Aravind and Koonin, 1999). Interestingly, the presence of these domains in a single protein is a typical feature of a substrate adaptor for CRL3, in which the BTB domain mediates interaction with Cullin 3, while the ankyrin repeat recruits substrates for ubiquitination (Petroski and Deshaies, 2005). However, yeast two-hybrid studies were unable to find direct physical interaction between Cullin 3 and NPR1 (Dieterle et al., 2005). Co-immunoprecipitation experiments nevertheless showed that NPR1 associates with a CRL3 *in planta* (Spoel et al., 2009). These results suggested that NPR1 may not be in the substrate adaptor position of this E3 ligase. Indeed, in *Arabidopsis* cells, monomeric NPR1 is itself subject to ubiquitination by a CRL3 and is subsequently degraded in the nucleus. Blocking NPR1 degradation pharmacologically with proteasome inhibitors or genetically by mutation of Cullin 3 resulted in accumulation of NPR1 monomer, moderate induction of NPR1 target genes, and elevated resistance to pathogen infection (Spoel et al., 2009). This indicated that constitutive degradation of NPR1 monomer by CRL3 prevents autoimmunity in absence of a pathogen threat. This suppressive effect of CRL3 and the proteasome

probably impacts a large proportion of the immune transcriptome, as many genes are co-regulated by SA and proteasome inhibitor (Spoel et al., 2010).

Ubiquitin-mediated protein degradation plays a similar role in SA-dependent immune responses in rice. Analogous to the function of *Arabidopsis* NPR1, *Oryza sativa* WRKY45 is an SA-induced transcription activator of several hundred immune-related genes and confers resistance to bacterial and fungal pathogens (Shimono et al., 2007, Shimono et al., 2012, Nakayama et al., 2013). Inhibition of the proteasome resulted in accumulation of polyubiquitinated OsWRKY45 in the nucleus and constitutive activation of its target genes in the absence of SA treatment (Matsushita et al., 2013). Although it remains unknown if *Os*WRKY45 is targeted for degradation by a CRL3, these findings indicate that constitutive turnover of this immune activator prevents autoimmune responses. SA also activates an NPR1-like protein, which functions in parallel with *Os*WRKY45 to regulate immune transcription in rice. By contrast to *Os*WRKY45, this *Os*NPR1 protein (also known as *Os*NH1) is thought to be predominantly involved in downregulation of gene expression, particularly those involved in photosynthetic activity (Sugano et al., 2010). Interestingly, *Os*NPR1 is not subject to constitutive proteasome-mediated degradation, intuitively suggesting that transcriptional repression does not require corepressor turnover. Hence, the presence of analogous proteasome-regulated modules consisting of unrelated transcription (co)activators in *Arabidopsis* and rice (*i.e.* NPR1 versus *Os*WRKY45) may reflect inherent constraints on how timely activation of SA-responsive immune genes can be achieved.

1.4.2. Ubiquitin-mediated activation of SA-responsive gene transcription

Besides suppression of SA-responsive immune genes, the proteasome is also involved in gene activation. Pharmacological inhibition of the proteasome, genetic mutation of Cullin 3, and mutations in a phosphorylation motif produce an increase in the stabilization of the NPR1 protein, leading to a greatly reduced the expression of SA-induced, NPR1-dependent immune genes in *Arabidopsis* (Spoel et al., 2009). Similarly, SA-induced transcriptional activity of *Os*WRKY45 in rice was impaired in the presence of proteasome inhibitor (Matsushita et al., 2013). Turnover of *Os*WRKY45 was dependent on a small 26 amino acid C-terminal region, which importantly was also required for its transactivation activity. Such overlap between transactivation domains and degradation motifs that signal ubiquitin-mediated proteasomal degradation has previously been discovered in transcription activators in both yeast and mammals (Salghetti et al., 2000). Fusion of well-defined degron motifs from yeast cyclin proteins to a DNA-binding domain even auto-activated gene transcription (Salghetti et al., 2000), suggesting that the intrinsic ability to activate transcription also makes activators a target for the ubiquitin-mediated proteasome. Subsequent work showed that like NPR1 and OsWRKY45, additional activators required turnover to unleash their full transcriptional potential (Geng et al., 2012, Spoel et al., 2010). This transcription process, sometimes dubbed 'destruction-activation', has been studied in more detail for general control non-inducible 4 (GCN4), a potent activator of genes involved in amino acid homeostasis. Upon amino acid starvation, the cell division cycle 4 (CDC4) F-box subunit of Skp, Cullin, F-Box (SCF^{CDC4}) ligase targets GCN4 for ubiquitin-mediated degradation, a process required for recruitment of RNA Polymerase II (RNAPII) to GCN4 target genes (Lipford et al., 2005). Crucially, GCN4 was marked for degradation by the

phosphorylative action of Suppressor of SNf1 10 (SRB10), a cyclin-dependent-kinase associated with the C-terminal domain of RNAPII (Chi et al., 2001, Liao et al., 1995). This indicates that when GCN4 initiates a round of transcription by recruiting RNAPII, it simultaneously triggers its own destruction. These results have led to the hypothesis that transcriptionally 'spent' activators may need to be cleared by the proteasome to reset target promoters and allow binding of 'fresh' activators (Figure 1.3) (Lipford et al., 2005, Kodadek et al., 2006, Geng et al., 2012). A similar mode of regulation may control transcriptional activity of NPR1 and OsWRKY45 in plant immunity, as site-specific phosphorylation of a degron motif in NPR1 was necessary for its ubiquitination and degradation, as well as for timely and sustained target gene expression (Spoel et al., 2009, Spoel et al., 2010). Intriguingly, transcription initiation by MYC2, a transcription activator responsive to the developmental and immune hormone jasmonic acid, is also regulated by phosphorylation-induced proteasomal degradation (Zhai et al., 2013). These findings imply that proteasome-mediated regulation of transcription activators may be a general mechanism to control gene expression programs in plant immunity.

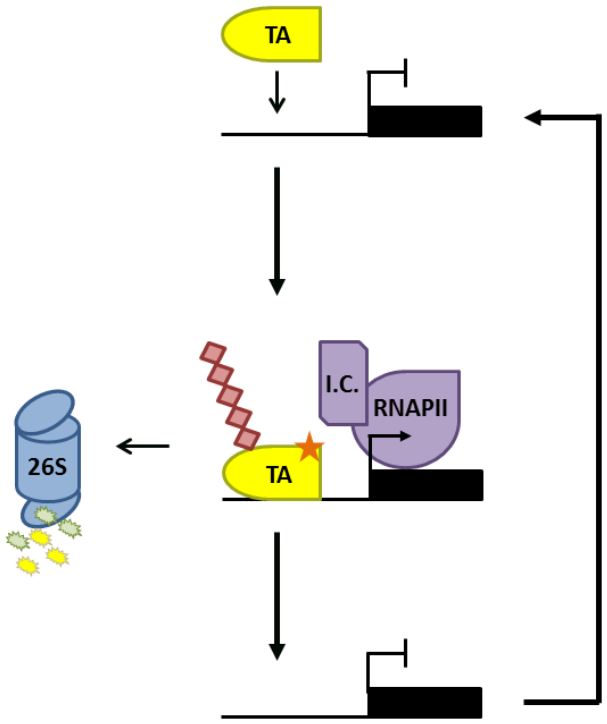

Figure 1.3: Proteasome-mediated activator turnover activates transcription.

Promoter binding of a transcription activator (TA) results in recruitment of the transcription initiation complex (I.C.) and RNAPII. The TA is subsequently phosphorylated (orange star) by a kinase within the I.C., marking it for ubiquitination (red diamonds) and degradation by the 26S proteasome. This allows a new TA to bind the promoter and reinitiate a new round of gene transcription.

Elegant studies on the estrogen receptor ERα in mammalian cells have shed more light on why activators are turned over in the process of activating gene transcription. Upon ligand binding, nuclear localized ERα forms a stable dimer and associates with cofactors on estrogen-responsive DNA elements to trigger gene transcription. Not only did inhibition of ERα proteolysis suppress its transcriptional activity, *vice versa* inhibition of RNAPII prevented degradation of ERα, indicating that activator turnover and transcriptional activity were interdependent (Reid et al., 2003). By following ERα transactivation over fine time scales by chromatin immunoprecipitation, it was proposed that ERα-mediated transcription

may have distinct cyclical phases in which the ubiquitin-mediated proteasome plays key roles (Metivier et al., 2003). In this model, the first cycle is transcriptionally non-productive but results in ERα-induced remodeling of the promoter to commit it to transcription. In subsequent cycles ERα orchestrates the ordered recruitment of cofactors, ultimately resulting in gene transcription via recruitment of RNAPII. Importantly, experimental data showed that the proteasome was recruited to an ERα target promoter towards the end of each cycle and preceded the clearance of ERα and general transcription cofactors. Thus, proteasome activity is thought to be vital to allow ERα-dependent promoters to move from the transcriptionally non-productive to productive phase and to permit productive cycles to continue until transcription is no longer required (Metivier et al., 2003, Zhou and Slingerland, 2014). If these findings indeed represent a general model for transcription regulation, then the proteasome could have additional roles in SA-responsive gene transcription in plants, including promoter remodeling and ordered cofactor degradation.

But why would cyclical activation of transcription by unstable activators be advantageous over continuous activation by stable activators? Although the answer to this question remains at large, a recent mathematical and *in silico* analysis of proteasome involvement in transcription may have provided some clues (Lee et al., 2014). The gene targets of many mammalian transcription activators often include components of E3 ligases that promote proteolysis of that activator, generating a negative feedback loop to maintain appropriate levels of activator. Mathematical modeling of this feedback loop showed that cellular perturbations resulting in destabilization of the E3 ligase led to over-accumulation of activators and subsequent hyper-activation of gene expression. However, if the E3 ligase was modeled as a necessary transcription cofactor working in conjunction with the activator, a much more measured gene expression output was achieved upon cellular perturbation. These models suggest that the paradoxical involvement of E3 ligases in gene

transcription activated by unstable activators may be necessary to provide a cellular safety mechanism. The authors of this work compared this to the principle of safety interlock devices in engineering, where a system will not function unless safety can be guaranteed (Lee et al., 2014). A similar system may be operational for NPR1- and OsWRKY45-dependent gene expression. Notably, interrogation of a list of NPR1-dependent genes provided by Wang et al. (2006) indicates that NPR1 activates the expression of genes encoding for its paralogues, NPR3 and NPR4. These BTB-containing proteins function as substrate adaptors that recruit NPR1 to CRL3 for ubiquitination and subsequent degradation (Fu et al., 2012). This suggests that similar to the mathematical system described above, a negative feedback loop may exist between NPR1 and $CRL3^{NPR3/NPR4}$. As CRL3 has a supportive role in NPR1-dependent gene transcription (Spoel et al., 2009), it may be part of a cellular safety mechanism to keep NPR1 activity in check when cellular perturbations are encountered. In support of this hypothesis, although genetic perturbations of $CRL3^{NPR3/NPR4}$ activity resulted in autoimmune phenotypes due to over-accumulation of NPR1 protein, this did not lead to over-activation of NPR1 target genes in the presence of SA (Spoel et al., 2009, Fu et al., 2012).

1.5. Aims and Objectives

Taken together, the above described literature clearly indicates that polyubiquitination is a highly important protein modification required to regulate protein function and degradation. It is crucial for the regulated destruction of transcription factors and a therefore essential for correct cellular gene expression. Although it may seem wasteful to constantly degrade functional proteins, direct coupling of the UPS to transcription may create a mechanism that combines tight regulation of TF activity while providing a safety net to prevent unwanted, spurious transcription.

Past research has focused mainly on polyubiquitination as a means to TF destruction, however it remains largely unknown if the (poly)ubiquitinated substrate is transcriptionally active or if (poly)ubiquitination itself alters TF performance. As E4 ligases enhance polyubiquitination, we reasoned that they may be able to inform us how the process of ubiquitination could affect TF activity. Work described in this thesis aimed to identify E4 ligases in *Arabidopsis* and investigate how they affect gene transcription and immunity orchestrated by the immune signal SA and the NPR1 coactivator. To find putative *Arabidopsis* E4s we search for ubiquitin ligases similar in homology to the yeast E4 ligases, (ubiquitin fusion degradation 2) UFD2 and (HECT ubiquitin ligase 5) HUL5, and screened these genes for global cellular polyubiquitination activity and specific polyubiquitination activity on NPR1. Furthermore, we studied if a role of E4 ligases in gene transcription may be conserved amongst eukaryotes. Because E4 ligases were first described in yeast, we investigated the role of two canonical E4 ligases in the yeast amino acid synthesis pathway of which the central transcription activator, GCN4, requires proteasome-mediated turnover to regulate gene expression. The information gathered by these experiments allowed us to further unravel the intimate link between transcription initiation and ubiquitination.

Chapter 2: Methods

2.1. Plant growth conditions

Soil: All plants were subject to the same growth conditions. Seeds were sown directly on soil containing a mix of peat moss, vermiculite and sand at a ratio of 4 : 1 : 1 and stratified by placing in a cold room for two days at 4 °C. Seeds were then moved into growth chambers at 21 °C and 100 µmol.m^{-2}.s^{-1} light on a 16-h-light/8-h-dark photoperiod at 65 % day humidity and 55 % night humidity. After ≈ 2 weeks of growth seedlngs were transplanted into new soil and kept in the same conditions.

Sterile medium: Approximately 50 µl of seeds were immersed in 1 ml of ddH_20 for 30 min before sterilization. To sterilize the seeds 1 ml of 100 % ethanol was added for 5 min, with regular vortexing. Then ethanol was then removed and 1 ml of 50 % bleach + 0.1 % triton added. Seeds were placed on a rotator for 30 min. and then washed 5 times in 1 ml sterile ddH_2O and stored in 1 ml sterile 0.1 % agar for 2 days at 4 °C. For growth, 25 seeds were placed onto an MS agar plate (Murashige and Skoog, 1962) containing 1x Gamborg's B5 (Sigma-Aldrich) vitamin solution. Plates were placed upright and grown for 12-16 days in the same light conditions as described above.

2.2. Plant genotypes

The Col-0 ecotype was used as wild type and all mutants and transgenics were prepared in this background. The *npr1-1*, *cul3a cul3b, sid2-2*, *35S::NPR1-GFP* (*npr1-1*) plants have been described previously (Kinkema et al., 2000, Spoel et al., 2009, Wildermuth et al., 2001). T-DNA insertion lines (Table 2.1) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The presence of the T-DNA was confirmed via genotyping using gene specific primers and a primer specific to the insertion. Knock-out of gene expression was confirmed via RT-PCR using gene specific primers (Supplemental table 2.1).

Table 2.1: T-DNA insertion mutant lines used in this study.

Locus	Allele	SALK/SAIL line
At5g15400	*ube4-1*	SALK-100087
	ube4-2	SAIL_713_A12
At4g38600	*upl3-1*	SALK_035524
At5g02880	*upl4-1*	SALK_091246C
	upl4-2	SALK_040984
At3g17205	*upl6-1*	SALK_055609C
	upl6-2	SALK_147660C
At3g53090	*upl7-1*	SAIL_403_A11
	upl7-2	SALK_119373(BU)

2.3. DNA extraction (Plants)

Tissue was homogenised in CTAB extraction buffer (100 mM Tris-HCl pH8, 1.5 M NaCl, 20 mM EDTA pH 8, 2 % CTAB [cetyl trimethylammonium bromide]) and heated at 65°C for 30 min. An equal volume of 24 : 1 chloroform : iso-amyl alcohol was added and samples centrifuged (13,000 rpm) for 5 min. This step was repeated with the aqueous layer. An equal volume of isopropyl alcohol was added to the aqueous solution and incubated for 1 h

at 4 °C. The precipitate was pelleted by centrifugation (15 min at 13,000 rpm), the supernatant was removed and the pellet washed in ice cold 70 % ethanol before being re-suspended in 50 µl of water.

2.4. RNA extraction (Plants)

Tissue was homogenised in RNA extraction buffer (100 mM LiCl, 100mM Tris (pH 8.0), 10 mM EDTA, 1 % SDS) to which an equal volume of phenol/chloroform/isoamylalcohol (25 : 24 : 1) was added. The homogenate was vortexed and then centrifuged (13,000 rpm) 5 min. The aqueous phase was transferred onto 1 volume of 24:1 chloroform/isoamylalcohol, vortexed and then centrifuged (13,000 rpm) 5 min; this step was repeated once. The aqueous layer was added to 1/3 volume of 8 M LiCl and incubated overnight at 4°C. The extract was then centrifuged (13,000 rpm) 5 min at 4°C. The resulting pellet was washed in 70 % ethanol (-20°C). The pellet was rehydrated in 400 µl of ddH_2O for 60 min on ice, re-suspended, and then 40 µl of NaAc (pH 5.3) and 1 ml 96 % ethanol (-20°C) added before incubating for 1 h at -20°C. The precipitate was then centrifuged (13,000 rpm) 5 min at 4°C, the pellet was rinsed in 70 % ethanol (-20°C) and re-suspended in 25 µl of water.

2.5. cDNA synthesis

RNA concentration was determined using a Nanodrop spectrophotometer and appropriate dilutions were made for equal RNA concentrations. RNA was reverse transcribed into cDNA using SuperScript Reverse Transcriptase II (Life Technologies) following the manufacturer's instructions.

2.6. RT-PCR

To confirm if T-DNA insertion knocked out the gene of interest RT-PCR was carried out using gene specific primers (Table2.2) and the products separated and visualised on a 1 % agarose gel.

Table 2.2 Primers used for RT-PCR to confirm knockout of specific genes.

Target gene	Target allele	Primer name	Primer Direction	Primer sequence 5'-3'
UBE4	ube4-1	UFD-F	F	CATGAGGCCCAAATATTGAGGGATG
		UFD-R	R	GGCAATGTTGTGGCGAATATTGAAC
	ube4-2	UFD2 TDNA F	F	ACCGAAATGCATGGAGACGG
		UFD2 TDNA RP	R	GCAGTGATCTCCTCGGATGT
UPL3	upl3-1	upl3-1 F	F	GGAGTTATCTCTCTTCCTACTAGCAC
		upl3-1 R	R	GTTAAGCAATGCTGCAACCACACCAC
UPL4	upl4-1	upl4-1 F	F	CAGGATTACCAGAGGCAGAGATC
		upl4-1 R	R	GACCACAGTAGAAATTGCGACTC
	upl4-2	UPL4-2 LP	F	CAGGCTGATTGACGAGAAAAC
		UPL4-2 RP	R	AGTACTTGGACGTTGCTGAGC
UPL6	upl6-1	upl6-1 F	F	GAACCGTTTAAGGGATCAGCTC
		upl6-1 R	R	AGCATTGGTTATCTGCTGCTCC
	upl6-2	upl6-2 F	F	TCAGAGGATGATCTACGGAGC
		upl6-2 R A	R	CGCAAGTCGTCTATATCCAAAC
UPL7	upl7-1	upl7-1A F	F	GGACCTGAATCGCAAACACAAG
		upl7-1A R	R	GTCGTAGAGCCAAAGTTACTGC
	upl7-2	upl7-2 F	F	CAAAACTGTTGGCAGCTTCTGG
		upl7-2 RP	R	AGATCCATGAGATGCATCGTC
UBQ10	N/A	UBQ10 F	F	GATCTTTGCCGGAAAACAATTGGAGGATGGT
		UBQ10 R	R	CGACTTGTCATTAGAAAGAAAGAGATAACAGG

2.7. qPCR

Quantitative-Real-Time-PCR (qPCR) was carried out using gene specific primer (Table2.3) on an Applied Biosciences Step-One-Plus RT-PCR machine (Life Technologies) as per the manufacturer's instructions.

Table 2.3: List of qPCR primers used in this study

Target gene	Primer Direction	Primer sequence 5'-3'
PR-1	F	CTAAGGGTTCACAACCAGGC
	R	AAGGCCCACCAGAGTGTATG
PR-2	F	CAGATTCCGGTACATCAACG
	R	AGTGGTGGTGTCAGTGGCTA
PR-5	F	ACTGTGGCGGTCTAAG
	R	CGTGGGAGGACAAGTTT
WRKY18	F	AGAAGGTACAACGCAGCGCAGA
	R	TGCGTCCCTTCGTATGTCGCTACA
WRKY38	F	CCGGTTTACCGAACCACTTA
	R	GGCTTTCCTTCTCCTGATCC
WRKY62	F	GCCTACACCAAGGACCAGAA
	R	AGAGGTGGAGGAGGAGAAGC
UBQ5	F	CCAAGCCGAAGAAGATCAAG
	R	ACTCCTTCCTCAAACGCTGA
ACT1	F	TTGGCCGGTAGAGATTTGAC
	R	AGTCCAAGGCGACGTAACAT
ADH5	F	TCGTTGGATCTTGTGTTGGA
	R	TTCAGGAACATCCGATAGGC
ARG1	F	CTCCGGACCAACCACAAGATT
	R	TAAGTTGGATGCGGCCAAGA
ASN1	F	ACTGCAAAGGCCACTAACGA
	R	TTGTCCAACCAGCAGTGTGT
CPA2	F	GTGCGGCAATGAGTGTTGTT
	R	TATAAGCAACGGCGTCCACA
HIS4	F	TCCAACCAATATGCACCAGA
	R	CTTGAATAGTCACCGCACGA
HUL5	F	GCCTACGGCATCTACCTGTG
	R	CTGGCGCCTGAGTTTATTGC
UFD2	F	TCCACGTCTGGGATCGTTTG
	R	AGGCATAGCACCAACGCTTA

2.8. Enhanced Disease Resistance (EDR) test

Twelve plants, aged 3-4 weeks, for each genotype were selected, half of which were sprayed with 0.5 mM SA and the other half mock sprayed with water. Plants were then left for 24 h before infiltration with the virulent bacterial pathogen *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326.

Bacteria were grown over night in liquid King's B medium. Cells were collected by centrifugation and diluted to OD 0.005 in 10 mM $MgCl_2$. The bacteria were pressure infiltrated via syringe into the abaxial surface of 2 leaves per plant; the plants were left for the time periods specified in figure legends before the infiltrated leaves were collected. The leaves were ground in 10 mM $MgCl_2$ at RT, the resulting solution was then serially diluted and 10 µl of each dilution was streaked onto LB agar containing 10 mM $MgCl_2$, Streptomycin 100 µg/ml, and 100 µM Cycloheximide. Plates were incubated at 30 °C for 2 days before colonies were counted at an appropriate dilution. Results were analysed with 95 % confidence intervals and via Tukey-Kramer ANOVA test ($\alpha = 0.05$, $n = 8$)

2.9. Enhanced Disease Susceptibility (EDS) test

Carried out as per EDR tests except that plants were not pre-treated with SA and the concentration of Psm ES4326 infiltrated into leaves was OD 0.0005.

2.10. Crude protein extraction and Western blots

Protein extractions and Western blots were performed as described previously (Spoel et al., 2009). Antibodies used in Western blots are detailed in figures.

2.11. Ubiquitinated protein pull-down assays

Seedlings of 12-16 days old were placed in solutions containing vehicle (DMSO), 0.5 mM SA and vehicle, or 0.5 mM SA and 100 µM MG132 for 6 h. Samples were weighed then frozen in liquid nitrogen. For analysis samples were ground in 2 volumes of protein extraction buffer (1X PBS, 1 % Triton X-100, 10 mM N-ethylmaleimide (NEM), Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich), protease inhibitor cocktail (TPCK 50 µg/ml, TLCK 50 µg/ml, 0.5 mM PMSF, 40 µM MG132) and 0.2 mg/ml recombinant GST-tagged Tandem Ubiquitin Binding Entities (TUBE) (Hjerpe et al., 2009). Homogenates were centrifuged for 20 min at 13,000 rpm at 4°C, the supernatant placed in clean tubes and centrifuged again. The lysate was filtered through 0.22 µM syringe driven filters and 30 µl was removed to act as input sample. The input samples were then incubated at 80 °C for 10 min in the presence of 1x SDS-PAGE sample buffer supplemented with 50 mM DTT. A total of 50 µl/ml of packed Protino® Glutathione Agarose 4B (Machery Nagel) was added to each sample and incubated at 4 °C overnight with rotation. Beads were collected by centrifugation for 20 s at 5,000 rpm at 4 °C and washed 5 times in protein extraction buffer without NEM. Samples were eluted from beads by the addition of 1X SDS-PAGE sample buffer supplemented with 50 mM DTT buffer and incubation at 80 °C for 10 min.

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2.12. Recombinant genes

UBE4 was cloned from cDNA (Primers in Table 2.4) and ligated into the Gateway pENTR/D-TOPO entry vector (Life Technologies) and transformed into TOP-10 chemically competent cells (Life Technologies). To generate *35S::YFP-UBE4* the *UBE4* containing entry vector was recombined with the Gateway compatible destination vector pEarleyGate 104 (Earley et al., 2006) using LR Clonase (Life Technologies) following the manufacturers' protocol and transformed into TOP10 cells. The *Agrobacterium tumifaciens* strain GV3101 (pMP90) was then transformed with the *35S::YFP-UBE4* construct using the freeze-thaw method (Weigel and Glazebrook, 2002). Transgenic plant lines were produced via the floral dipping method (Weigel and Glazebrook, 2002).

Table 2.4: Primers used to clone UBE4 from cDNA

Target gene	Primer name	Primer Direction	Primer sequence 5'-3'
UBE4	UFD2 TOPO F	F	CACCATGGCGACGAGCAAACCTCAAAG
	UFD2 Blunt RP	R	TTAATCAATTAACATATCACTG

2.13. UBE4 localisation

Protoplasts were isolated from 3 – 4 week old plants using the tape-sandwich method (Wu et al., 2009), and were transformed with the pEarleyGate 104 plasmid carrying *35S::YFP-UBE4*. Protoplasts were then resuspended in W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 2 mM MES, pH 5.7) and allowed to rest for 18 hours in the light. YFP fluorescence was then assessed on a Nikon Eclipse TC2000-U confocal microscope.

2.14. Co-immunoprecipitation

Samples were ground in two volumes of protein extraction buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.1 % Triton X-100, 0.2 % Nonidet P-40, protease inhibitors [50 µg/ml TPCK, 50 µg/ml TLCK, 0.6 mM PMSF, 40 µM MG132], and 40 µM PR619 deubiquitinase inhibitor (Abcam)). Homogenates were centrifuged twice (13,000g) at 4 °C for 20 min. The lysate was filtered through 0.22 µM syringe driven filters and pre-cleared for 1 hour with Protein A agarose beads (Millipore). An aliquot of 30 µl was removed as input samples, which were then incubated at 80 °C for 10 min in the presence of 1x SDS-PAGE sample buffer supplemented with 50 mM DTT. The remaining samples amounts were incubated with α-GFP (Roche) or α-ab290 (Abcam) at a concentration of 1 : 500 for 2 hours at 4 °C with gentle rocking. Beads were collected by brief centrifugation (6,000 rpm) and washed 5 times with extraction buffer and eluted by the addition of 1x SDS-PAGE sample buffer supplemented with 50 mM DTT buffer and incubation at 80 °C for 10 min.

2.15. RNA-seq

Three-week old plants were sprayed with 0.5 mM SA or mock sprayed with water. Two leaves from 6 plants were collected per sample. RNA was extracted as a described above, then purified via RNeasy Mini Kit (Qiagen) using the RNA clean up protocol as described by the manufacturer, to remove residual phenol. qPCR analysis was carried out to confirm appropriate induction of SA responses. Samples were then quantified and submitted to GATC Biotech (Constance, Germany) for RNA sequencing and bio-informatic analysis.

2.16. Cis-promoter analysis of UPL3 dependent genes

To generate the list of SA-mediated and UPL3- dependent genes, we compared those genes with twofold or greater change in expression after SA induction in wild type, against a list of genes with a 1.5-fold difference in expression between wild type and *upl3* after SA induction. We then applied a p-value of < 0.05 and a q-value cut-off of < 0.01 to filter out false positives. All possible 8 bp (octamer) combinations were first calculated to obtain relative appearance ratio, comparing the promoter regions of selected gene set against all genes in *Arabidopsis*. The most enriched pentamers were subsequently calculated from the octamers. Weblogo analysis (Crooks et al., 2004) was performed using pentamers plus the adjacent sequences in the selected gene set. Cis-promoter and weblogo analysis was performed in collaboration with the laboratory of Prof. Yasuomi Tada Nagoya University.

To investigate if the discovered motives were more or less prevalent in the UPL3-dependent gene sets, the first 1000 bp upstream of the TAIR 10 loci of the top 55 genes with the greatest up- and down-regulated expression change were analysed with the POBO promoter analysis tool (Kankainen and Holm, 2004).

2.17. Yeast deletion mutants

Wild type yeast and E4 ligase mutants were ordered from the EUROSCARF stock centre (Table 2.5) (Euroscarf, 2015). Mutants were genotyped to confirm deletion using primers in supplemental table 2.2.

Table 2.5: Euroscarf yeast accessions used in this study

	Accession	Genotype
Wild type	Y00000	BY4741; mat a; his3Δ 1; leu2Δ 0; met15Δ 0, ura3Δ 0
ufd2Δ	Y03888	BY4741; mat a; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; YDL190c::kanMX4
hul5Δ	Y04508	BY4741; mat a; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; YGL141w::kanMX4

2.18. DNA extraction (Yeast)

Yeast DNA was extracted via the Bust n' Grab protocol (Harju et al., 2004).

2.19 RNA extraction (Yeast)

Pelleted cells were re-suspended in 400 µl AE buffer (50 mM NaAc (pH 5.3), 10 mM EDTA) and 40 µl of 10 % SDS. Samples were vortexed before the addition of an equal volume of buffered phenol solution (pH < 8). The samples were incubated at 65 °C for 30 min with regular vortexing. Samples were then subjected to 2 freeze/thaw cycles before being centrifuged (13,000 rpm) for 10 min. The aqueous phase was removed and added to 400 µl phenol : chloroform (5 : 1, pH 4), vortexed, incubated at RT for 5 min, and then centrifuged (13,000 rpm) for 5 min. The aqueous phase was removed, added to 400 µl chloroform and centrifuged (13,000 rpm) for 5 min. The aqueous phase was added to an equal volume of isopropanol, placed on ice for 5 min and centrifuged (13,000 rpm) for 5 min. Supernatant was removed, the pellet washed in 80 % ethanol and re-suspended in 25 µl ddH_20. RNA was subsequently treated with TurboDNA-free DNase (Life Technologies) as per manufacturer's instructions.

2.20. Protein extraction (Yeast)

For Western blotting protein was protein was extracted by mild alkali treatment and boiling in SDS sample buffer as described previously (Kushnirov, 2000).

2.21. Amino acid starvation assays (Yeast)

Yeast was cultured overnight at 28 °C in SMM (Supplemented Minimal Media: 0.67 % Yeast Nitrogen Base without amino acids, 2 % glucose and amino acids that the strains were auxotrophic for) and then diluted to OD 0.2 in 20 ml SMM and allowed to grow to mid-log phase (OD 0.5-1).

Subsequently, 10 ml of culture was removed as the non-induced control, and cells were collected by centrifugation at 4500 rpm for 10 min. Media was removed and the cells were washed in water, collected by centrifugation, and the pelleted cells frozen in liquid nitrogen and stored at -80 °C.

The cells in remaining 10 ml of culture were collected by centrifugation and re-suspended in SMM without leucine to induce amino acid synthesis. Cells were collected as described above at indicated time points.

2.22. GCN4 Myc-tagging

A 13-myc tag was applied to the C-terminal of GCN4 using the pFA6-13Myc-HisMX6 construct (Longtine et al., 1998) with primer over-hangs homologous GCN4 (Table 2.5). This construct was directly transformed into yeast cells as described below, genotyped with

construct-specific primers (Table 2.6) to confirm construct presence, and then western blotted to confirm GCN4-13Myc expression.

Table 2.6: Primers used to clone the 13-Myc construct used to tag GCN4.

Target gene	Primer name	Primer Direction	Primer sequence 5'-3'
GCN4	GCN-F2	F	TTGGAAAATGAGGTTGCCAGATTAAAGAAATTA-GTTGGCGAACGC*CGGATCCCCGGGTTAATTAA*
	GCN-F1	R	ATTTCGTTATACACGAGAATGAAATAAAAAATATAAA-ATAAAAGGTAAATGAAA*GAATTCGAGCTCGTTTAAAC*

Note: Non-italics indicate primers which overlap GCN4. Italics indicate bases which overlap the pFA-13Myc construct.

Table 2.7: Primers used to confirm presence of GCN4 -13Myc in transformed yeast cells.

Target gene	Primer name	Primer Direction	Primer sequence 5'-3'
GCN4-13Myc	GCN4 LP	F	CTTTCTCCAATTGTGCCCGAATCC
	RpTef	R	GGATGTATGGGCTAAATGTAC

2.23. Yeast transformation

Yeast culture was grown overnight in YPD, diluted in 50 ml YPD to OD 0.2, and grown to mid-log phase. Cells were collected by centrifugation (6,000 rpm) for 5 min. Cells were re-suspended and washed in 10 ml ddH_20, collected and washed in 1 ml 0.1 M LiAc, and then washed again in 250 µl 0.1 M LiAc. Subsequently, 50 µl of cells were re-suspended in transformation buffer (240 µl 50 % polyethylene-glycol, 20 µl Salmon sperm DNA, 36 µl 1 M LiAc, 20 µl DNA construct), rotated for 45 min at 30 °C, and then heat shocked at 42 °C for 15 min. Cells were collected and re-suspended in 300 µl ddH_20, then plated onto appropriate selective media.

2.24. GCN4 turnover

Cultures were grown overnight in SMM +Leucine and diluted to OD 0.2. Cultures were then grown to mid-log phase before switching cells into SMM without Leucine to induce starvation. After 1 h 100 mM Cycloheximide was added and samples taken at indicated time-points.

2.25. Amino acid induction with proteasome inhibitor

Yeast was cultured overnight at 28 °C in a synthetic medium (0.17 % Yeast Nitrogen Base without amino acids and ammonium sulphate, 0.1 % L-proline, 2 % glucose and amino acids that the strains were auxotrophic for), diluted to OD 0.2 in 30 ml of media containing 0.003 % SDS, and allowed to grow to mid-log phase (OD 0.5-1). The culture was then divided into 10 ml cultures that were treated with 75 µM MG132 (dissolved in DMSO) or DMSO. Cultures were incubated for 30 min and cells collected by centrifugation. One DMSO treated culture was stored as an un-induced control, while the remaining cultures were re-suspended in the same medium supplemented with 75 µM MG132 or DMSO but without leucine. Finally cells were collected after 2 h for further analysis.

Chapter 3: Processive ubiquitination controls NPR1 coactivator activity in plant immunity

3.1. Introduction

As outlined in the introduction, plant defences against biotrophic pathogens are regulated by the hormone salicylic acid (SA). Pathogen-induced SA levels mediate immune gene expression via the transcription cofactor NPR1 (Fu and Dong, 2013). Upon activation of NPR1 by SA, NPR1 moves into the nucleus (Tada et al., 2008, Mou et al., 2003, Kinkema et al., 2000), where a recent advance in NPR1 regulation has shown that the Small Ubiquitin-like Modifier (SUMO) is crucial to activation of NPR1-mediated transcription (Saleh et al., 2015). As well as promoting nuclear transfer of NPR1, SA induces dephosphorylation at NPR1 Serine 55 / Serine 59, via an unknown mechanism, which promotes sumolyation of NPR1 by SUMO3. This allows NPR1 to dissociate from the repressor WRKY70 and also induces the phosphorylation of NPR1 residues Serine 11 / Serine 15 that activate NPR1, promoting binding to TGA transcription factors to generate gene expression (Saleh et al., 2015). Counter-intuitively, activated NPR1 requires regulated degradation via the proteasome to maintain target gene expression. Blocking of NPR1 turnover via mutation of its phosphodegron, deletion of the Cullin 3 (CUL3) E3 ligase that ubiquitinates NPR1 to signal for its destruction, or chemical inhibition of the proteasome all reduce transcription of target genes (Spoel et al., 2009). This method of regulation may allow tight control of transcription by a combination of nuclear localisation, activation and degradation, with the destruction of NPR1 coactivator providing the ultimate control step. This model provides a direct link between the rate of transcription to the speed of NPR1 turnover. However, the model assumes that once NPR1 has activated a round of transcription via RNAP II, the Serine 11 / Serine 15 phosphorylated NPR1 is ‘exhausted’;

therefore 'fresh' NPR1 is required to execute further transcriptional cycles. Crucially, the idea that NPR1 earmarked for degradation is exhausted, i.e. that it is unable to initiate further rounds of transcription, cannot be proven with the pharmacological and genetic approaches used to date, as they either prevent initial ubiquitination events or block degradation of highly polyubiquitinated substrates. Thus, these methods do not reveal if ubiquitination of NPR1 in absence of proteasome-mediated degradation has any effects on its intrinsic transcriptional activity.

Within other eukaryotic systems the act of ubiquitination in itself without degradation has been shown to have an effect on the activity of TFs to initiate gene expression. Depending upon the TF and the position of the ubiquitin moiety, monoubiquitination can lead to enhancement or suppression of a TFs ability to activate gene expression. Suppression of TF activity can be achieved via several ways. The mammalian p53 tumour suppressor protein is monoubiquitinated by the E3 mouse double-minute 2 (MDM2) which signals for nuclear to cytoplasmic relocalisation, thereby preventing transcription (Wu et al., 2011). Monoubiquitination of mammalian Receptor-activated SMADs (R-SMAD), involved in transforming growth factor-β (TGF-β)-mediated embryonic development and tissue homeostasis, attenuated its transcriptional activity by two possible mechanisms: (*i*) monoubiquitination prevented either R-SMAD transcription complex formation or DNA binding by steric hindrance; or (*ii*) monoubiquitinated R-SMADs are actively removed from the promoter (Ndoja et al., 2014, Inui et al., 2011, Tang et al., 2011) Enhancement of transcription factors can be achieved as monoubiquitination can also stabilise TFs and prevent their removal from the promoter by the RP subunit of the proteasome allowing transcription to continue (Ferdous et al., 2007, Archer et al., 2008a, Archer et al., 2008b, Kim et al., 2009).

Monoubiquitination does not normally signal for degradation, the canonical signal for which is K48 linked ubiquitin chains of at least four moieties long. It is possible for monoubiquitination signals to be extended into polyubiquitin chains in which the TF may remain active, therefore the time required for ubiquitination and degradation of a TF could be used as a timer to provide a window in which a TF could be active (Wu et al., 2007). As ubiquitin can directly alter the activity of TFs and the activity of NPR1 has been shown to be regulated by the ubiquitin related molecule SUMO (Saleh et al., 2015), it is feasible that the similar act of ubiquitination in itself could have an effect on the ability of NPR1 to promote transcription. Using a genetic approach to dissect enzymes of the ubiquitination pathway, in this chapter we investigated how ubiquitination of NPR1 before its subsequent degradation could alter its transcriptional activity.

Within the ubiquitination pathway, it is the E3 ligases that recognise substrates and provide the link between the E2 conjugating enzyme and the substrate to initiate ubiquitin chain formation (Geng et al., 2012). Ubiquitin chain elongation can be enhanced by the E4 class of ubiquitin ligases, which, like E3 ligases, serve to act as the bridge between a substrate and the E2 (Tu et al., 2007). The first E4 ligase discovered was UFD2 in yeast. UFD2 was found to increase the efficacy of ubiquitination on substrates that had already been partially ubiquitinated, but UFD2 in itself could not perform the initial ubiquitination steps (Koegl et al., 1999, Saeki et al., 2004). UFD2 is not critical to yeast growth in normal unstressed conditions but deletion causes reduced ubiquitin chain length and compromised stress responses (Koegl et al., 1999). A second E4 HUL5 was subsequently discovered in yeast, acting as a proteasome-associated protein acting in opposition to the deubiquitinase ubiquitin-specific protease 6 (UBP6). Deletion of HUL5 led to an imbalance of ubiquitin ligase versus deubiquitinases activity, causing proteasome substrates to have decreased ubiquitin chain lengths which increased their stability (Crosas et al., 2006). The mammalian

homolog of UFD2, ubiquitin ligase E4 (UBE4), enhances the initial monoubiquitination mark on p53 performed by the E3 ligase MDM2, signalling p53 for degradation (Wu et al., 2011). As E4 ligase mutants have reduced levels of substrate ubiquitination and subsequent degradation (Koegl et al., 1999, Crosas et al., 2006) they therefore provide a platform in which to study the effect of the initial act of ubiquitination on a substrate.

In this chapter we used a homology search to the yeast E4 ligase, UFD2 (Koegl et al., 1999, Huang et al., 2014), and identified UBE4 as a potential E4 ligase in *Arabidopsis.* We found that like mutation of the E3 ligase CUL3, knockout of UBE4 produced a reduced level of polyubiquitination and turnover of NPR1. However, in marked contrast to *cul3* mutants, mutant *ube4* plants exhibited enhanced activation of SA-dependent genes that was uncoupled from proteasome activity. Our data provide the first evidence that CUL3-mediated ubiquitination of NPR1 renders it in a transcriptionally active form and that further UBE4-mediated polyubiquitination promotes proteasome-mediated turnover of NPR1, thereby establishing a limited window of opportunity for transcriptional activation.

3.2. Results

3.2.1. *Arabidopsis* UBE4 is homologous to the yeast E4 ligase UFD2

In order to discover *Arabidopsis* putative homologs of the yeast E4 ligase UFD2 (Koegl et al., 1999), a BLASTP search using the *S. cerevisiae* UFD2 protein sequence was initiated. The result of the search produced only one likely candidate gene, AT5G15400, which we designated as Ubiquitination factor E4 (UBE4) analogous to terminology of human E4 ligases. A structural homology modelling search using PHYRE2 (Kelley et al., 2015) confirmed that the protein structures of ScUFD2 and UBE4 are indeed homologous (e-value 1e-150) (Figure 3.1, Supplemental 3.1). These results agree with a previous analysis by Azevedo et al (2001) of plant U-box domain containing proteins, they found that *At*UBE4 (referred to as *At*UFD2 by Azevedo et al) was the closest plant homolog to *Sc*UFD2 and that *At*UBE4 was the single member of the five plant U-box protein classes (Appendix 3.1), providing evidence that UBE4 is the closest *Sc*UFD2 homolog in *Arabidopsis*. ScUFD2 has a three domain structure, consisting of a C-terminal U-box domain, a UFD2 core domain, and an N-Terminal Domain (NTD). The U-box domain is required for recruitment and association with E2 ubiquitin conjugating enzymes (Tu et al., 2007). The UFD2 core domain contains sequences similar to Armadillo (Arm) repeats found in α-importin, which bind nuclear localisation sequences, suggesting UFD2 is involved in protein-protein interactions (Tu et al., 2007). Indeed, the ScUFD2 core can act as a docking site for cell division cycle 48 (CDC48), which helps regulate ScUFD2 interaction with the proteasome shuttle protein RAD23 (Baek et al., 2011). RAD23 is bound by the unique NTD, which harbours a ubiquitin associated domain, providing the ability to recognize ubiquitin or ubiquitin-like domains (Hanzelmann et al., 2010, Tu et al., 2007). The recognition of ubiquitin or ubiquitin-like domains via the N-terminal domain of UFD2 and the binding of E2 ligases on the U-box is

thought to provide ScUFD2 with its E4 ligase function of extending pre-exisiting ubiquitin chains (Koegl et al., 1999, Saeki et al., 2004, Hanzelmann et al., 2010, Tu et al., 2007). As UBE4 is a structural homolog of ScUFD2, it is highly likely this protein serves the same function in *Arabidopsis*.

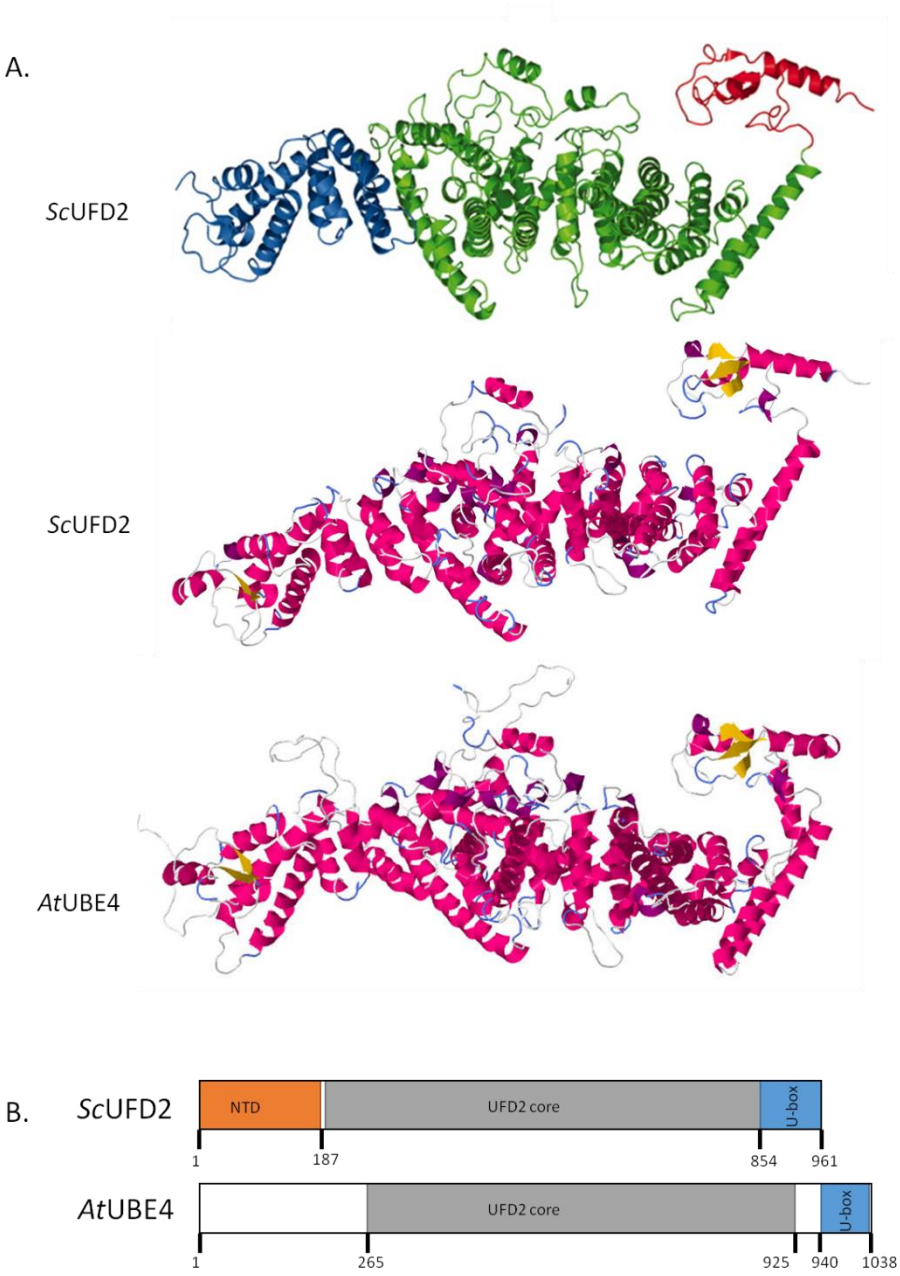

Figure 3.1: *Arabidopsis* UBE4 is a structural homologue of *S.c.*UFD2.

A. (Top) *Sc*UFD2 structure coloured by domain (Blue: N-Terminal Domain, Green: UFD2 core domain, Red: U-box. as derived from Tu et al (2007). **(Middle)** The *Sc*UFD2 protein structure used by PHYRE2 as the template for modelling UBE4. **(Bottom)** Predicted protein structure of *Arabidopsis* UBE4 generated by PHYRE2 from the UBE4 amino-acid sequence provided by TAIR. **B.** *ScUFD2* domain structure cartoon drawn from data from Tu et al (2007)*. At*UBE4 Domain structure cartoon drawn from results generated by InterPro supplied with the protein amino acid sequence from TAIR 10. Numbers indicate amino acid position. NTD: N-terminal Domain. Note: The NTD does not appear on *At*UBE4 cartoon as the domain is not recognised by Interpro.

3.2.2. Isolation of *ube4* knock-out mutant plants

To study the molecular and biological function of UBE4 in Arabidopsis, relevant T-DNA insertion mutants were ordered from the SALK mutant library (Alonso et al., 2003) and genotyped for gene expression of *UBE4*. Whereas the SALK T-DNA insertion mutant allele, *ube4-1* was not a functional gene knockout, the *ube4-2* allele contained a SAIL T-DNA at the 3' end of exon ten (Figure 3.2A), leading to knockout of *UBE4* gene expression and no UBE4 protein produced (Figures 3.2B & C). Compared to wild type plants, mutant *ube4-2* plants displayed wavy leaves and had a buckled appearance (Figure 3.2D), suggesting UBE4 also plays a role in plant development.

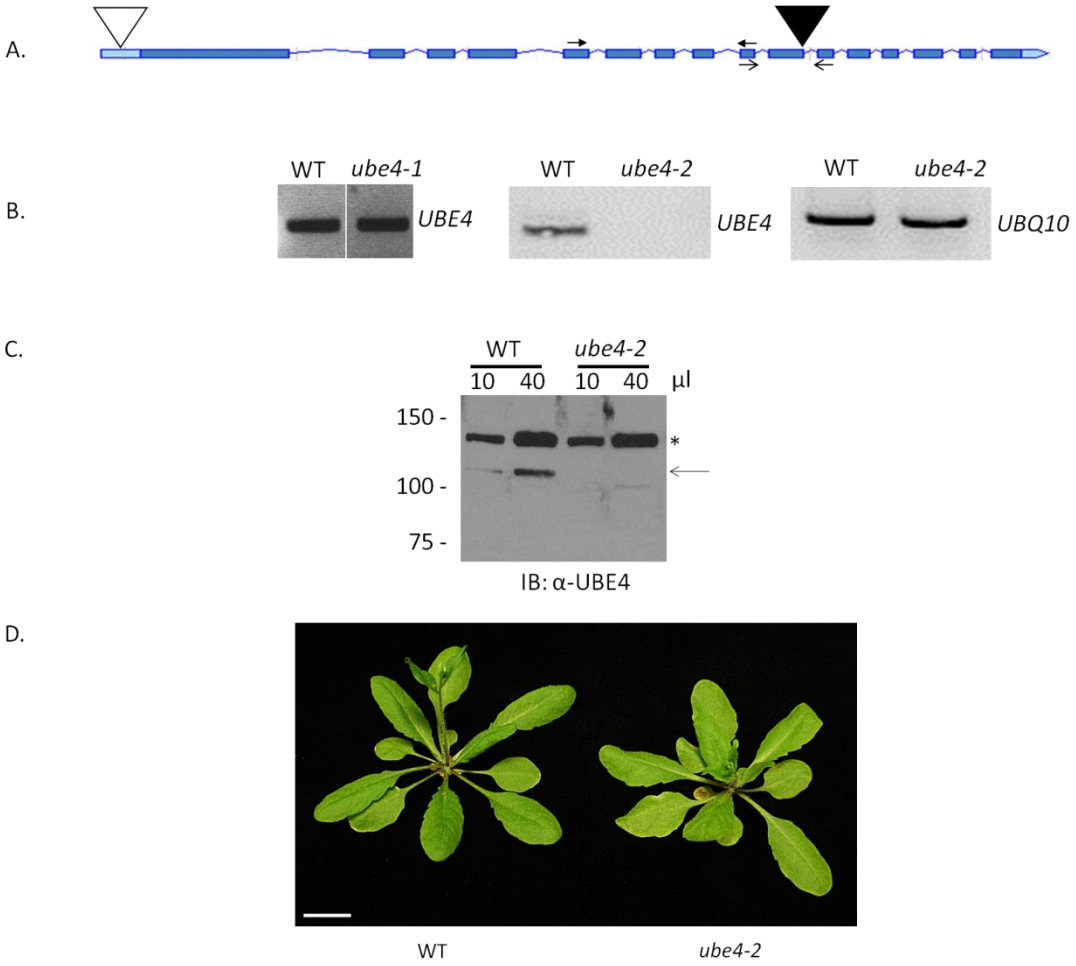

Figure 3.2: SAIL insertion knockout of *UBE4* leads to altered growth morphology.

A. *UBE4* (AT5G15400) gene structure. White triangle indicates the position of the SALK_100087 T-DNA insertion within the 5' UTR (*ube4-1*). Black triangle indicates the position of the SAIL_713_A12 T-DNA insertion at the 3' of exon 10 in (*ube4-2*). Closed arrowheads indicate locations of primers used for RT-PCR of *ube4*-1. Open arrowheads indicate locations of primers used for RT-PCR of *ube4-2.* Dark blue rectangles: exons. Lines: introns. Light blue rectangles: UTR. Image modified from Arabidopsis.org. **B.** RT-PCR of *UBE4* gene expression. A single leaf from six plants was collected and pooled for RT-PCR using *UBE4* gene-specific primers. **C.** Western blot of UBE4 protein in wild type and *ube4-2*. Arrowhead indicates UBE4. Asterisk indicates non-specific binding, used as a loading control. A single leaf from six plants was collected and pooled for protein extraction. Samples were probed with anti-UBE4. µl indicates amount of crude protein extract loaded. **D.** Morphology of 4-week old wild type (WT) and *ube4-2* plants. Scale bar 1 cm.

3.2.3. Mutant *ube4-2* plants exhibit autoimmunity

Knockout of UBE4 produced an obvious phenotypic effect, indicating it plays a functional role in *Arabidopsis*. Therefore we investigated if *ube4-2* mutants had differences in the basal regulation of SA-dependent genes compared to wild type. Seedlings were grown on MS-agar plates for 14 days before analysis of defence related marker genes via qPCR. All of the genes tested had elevated basal levels in the *ube4-2* mutant, suggesting that UBE4 performs a regulatory role in the expression of SA-dependent defence genes (Figure 3.3).

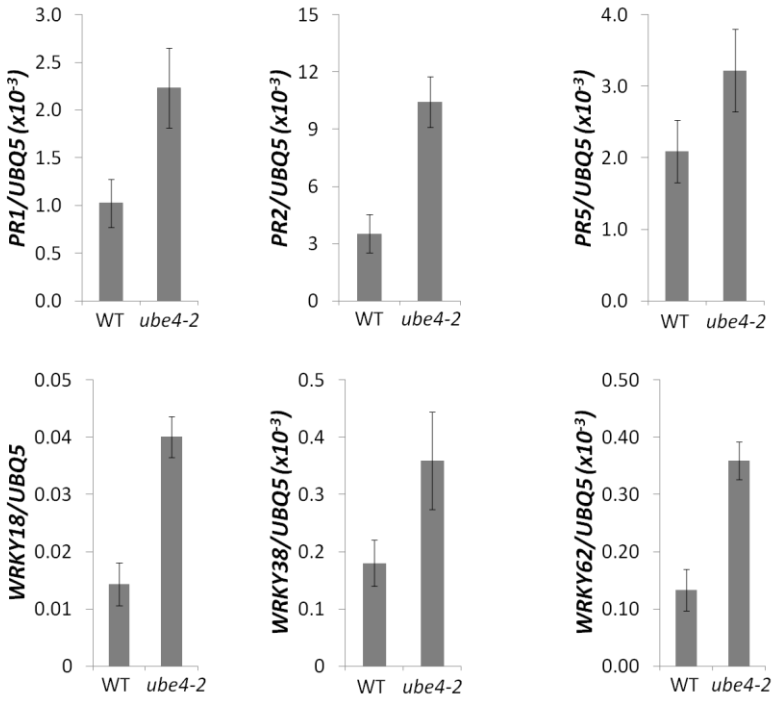

Figure 3.3: Knockout of UBE4 leads to increased basal levels of SA-dependent immune response genes.

14 day old seedlings grown on MS agar plates of each genotype were pooled and used for qPCR with gene specific primers against SA-responsive *PR-1, PR-2, PR-5*, *WRKY18*, *WRKY38*, and WRKY62. Expression was normalized against constitutively expressed ubiquitin 5 (UBQ5). n = 3. Error bars indicate standard error from 3 technical replicates.

As *ube4-2* plants showed elevated basal expression of SA-responsive immune genes, we assessed if this was associated with enhanced immunity. To that end we used the bacterial leaf pathogen *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326, which is resisted through the SA-dependent immune signalling pathway (Kloek et al., 2001). Twenty-four hours prior to inoculation with *Psm* ES4326, plants were sprayed with either water or SA to activate SAR. Wild type plants that had been sprayed with SA showed a significant reduction in pathogen growth and leaf chl-orosis. By contrast, mutant *npr1-1* plants were highly susceptible to *Psm* ES4326 and had no observable response to treatment with SA. Interestingly, water-treated *ube4-2* mutants already showed enhanced resistance to *Psm* ES4326 and attained the same level of resistance as wild type after SA treatment. Wild type plants that were pre-treated with SA showed less chlorosis than mock-treated ones. However, *ube4-2* mutants showed more chlorosis than wild type plants treated with or without SA (Figure 3.4). These data indicate that *ube4-2* mutants have enhanced basal disease resistance but that disease symptoms are uncoupled from pathogen growth.

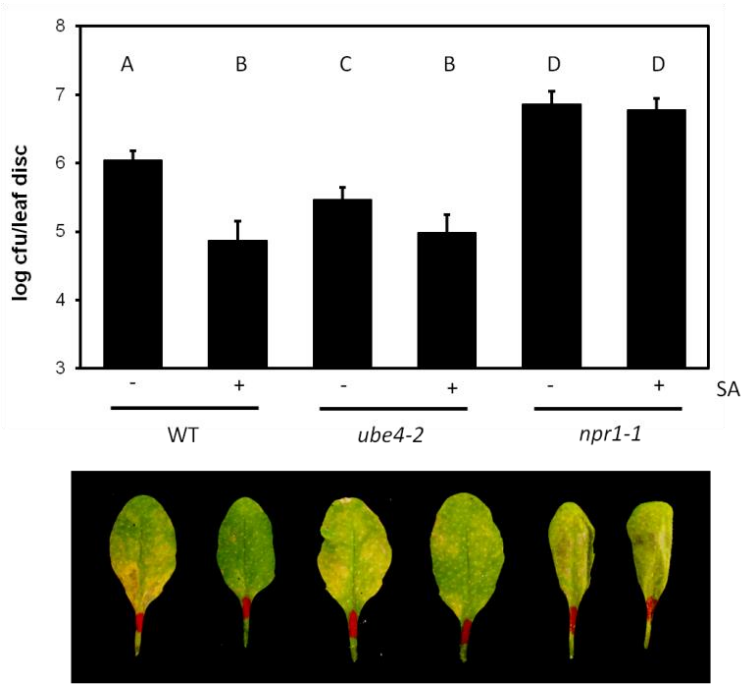

Figure 3.4: Mutant *ube4-2* plants exhibit enhanced basal disease resistance.

(Top) Quantification of pathogen levels within infected leaves. **(Bottom)** Disease symptoms of infected leaves. Three-week old wild type, mutant *ube4-2* and mutant *npr1-1* plants were sprayed with water (-) or 0.5 mM SA (+) and incubated for 24 hours, before syringe infiltration with 5 x 10^6 colony forming units/ml *Psm* ES4326 into the abaxial surface of a leaf. Leaf discs were collected 4-days post inoculation, bacteria were extracted and serial diluted before colony counting on selective agar plates. Error bars indicate 95 % confidence limits (n = 8). Letters indicate significantly different samples (Tukey-Kramer ANOVA test, $\alpha = 0.05$, n = 8).

3.2.4. Autoimmunity in *ube4-2* mutants is dependent on SA and the transcription coactivator NPR1

To investigate if autoimmunity and enhanced basal resistance displayed by *ube4* (Figures 3.3 & 3.4) is dependent on the defence hormone SA, the *ube4-2* mutant was crossed into the SA-deficient mutant *SA induction deficient 2-2* (*sid2-2)* (Wildermuth et al., 2001). Compared to the wild type, mutant *sid2-2* plants showed similar basal expression of SA-responsive *WRKY18* and *WRKY62*, but a reduction in basal expression of SA-responsive *PR1* and *WRKY38* was observed (Figure 3.5). As expected, *ube4-2* mutants had higher levels of expression for *PR-1*, *WRKY18* and *WRKY62*, and similar levels of WRKY38 relative to wild type. By contrast, these elevated gene expression levels were completely eliminated in the *ube4-2 sid2-2* double mutant (Figure 3.5), illustrating that enhanced gene expression in *ube4-2* mutants is dependent on SA production.

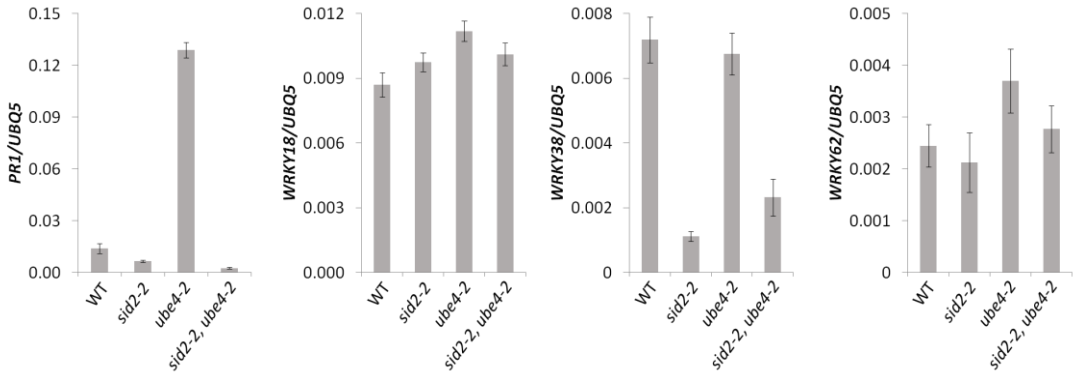

Figure 3.5: Mutant *ube4-2* plants display increased basal immune gene transcription that is dependent on SA signalling.

One leaf from six 3-week old adult plants was collected and used for qPCR with gene specific primers against SA-responsive *PR-1*, *WRKY18*, *WRKY38*, and WRKY62. Expression was normalized against constitutively expressed ubiquitin 5 (UBQ5). Bars indicate standard error from 3 technical replicates.

To test if enhanced immunity in *ube4-2* mutants was also dependent on SA, 3-week old plants were inoculated with a low dose (5 x 10^5 colony forming units/ml) of the virulent pathogen *Psm* ES4326 and incubated for 4 days. As expected, wild type and *ube4-2* plants showed similar levels of resistance to this pathogen despite *ube4-2* having generally higher defence gene expression. This is due to the low dosage of pathogen used, for which the basal level of defence gene expression in wild type in already enough to achieve maximal repression of pathogen growth and disease symptoms. Both the *sid2-2* single and *ube4-2 sid2-2* double mutants were more susceptible to disease as indicated by significantly higher pathogen growth and chlorosis of the leaf (Figure 3.6). These data demonstrate that regulation of defence gene by UBE4 is dependent on SA production and signalling.

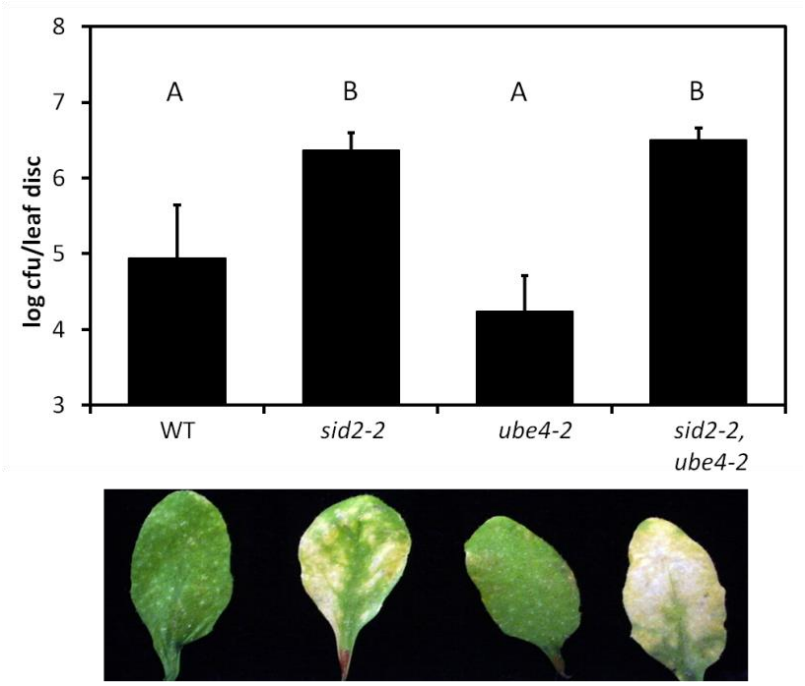

Figure 3.6: UBE4 requires SA to maintain basal immune defences.

(Top) Quantification of pathogen levels within infected leaves. **(Bottom)** Morphology of infected leaves. Three-week old adult plants were inoculated via syringe infiltration with 5 x 10^5 colony forming units (cfu)/ml *Psm* ES4326 into the abaxial surface of a leaf. Leaf discs were collected 4-days post inoculation, bacteria were extracted and serial diluted before colony counting on selective agar plates. Error bars indicate 95 % confidence limits (n = 8). Letters indicate significantly different samples (Tukey-Kramer ANOVA test, $\alpha = 0.05$, n = 8).

As NPR1 is the master regulator of the SA-dependent immune response, *ube4-2* was also crossed into the *npr1-1* mutant. As expected, the *npr1-1* mutant displayed reduced expression of several marker genes compared to wild type (Figure 3.7). Again the *ube4-2* single mutant showed increased expression of marker genes compared to wild type, except for WRK18, which was similar. The *ube4-2 npr1-1* double mutant, however, exhibited expression levels that were comparable to the *npr1-1* single mutant (Figure 3.7), indicating that the enhanced gene expression in *ube4-2* is dependent on the SA-mediated transcription co-factor NPR1.

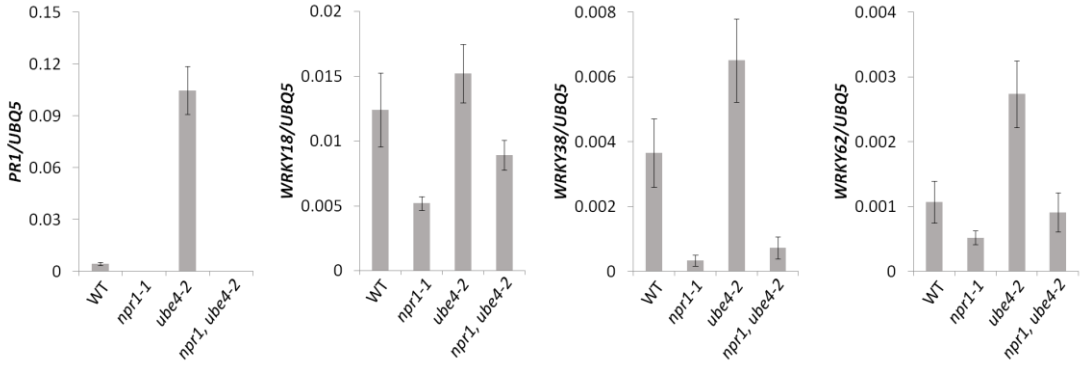

Figure 3.7: Mutant *ube4-2* plants exhibit increased basal immune gene transcription that is dependent on SA-responsive NPR1 coactivator.

One leaf from six 3-week old adult plants was collected and used for qPCR with gene specific primers against SA-responsive *PR-1*, *WRKY18*, *WRKY38*, and WRKY62. Expression was normalized against constitutively expressed ubiquitin 5 (UBQ5). Bars indicate standard error from 3 technical replicates.

To further investigate if enhanced immunity of *ube4-2* was also dependent on the SA-mediated co-factor NPR1, plants were inoculated with a low dose (5 x 10^5 colony forming units/ml) of the virulent pathogen *Psm* ES4326 and incubated for 4 days. Mutant *ube4-2* plants had equivalent resistance as the wild type, but in the *npr1-1* background had the same disease levels as the single mutant (Figure 3.8).

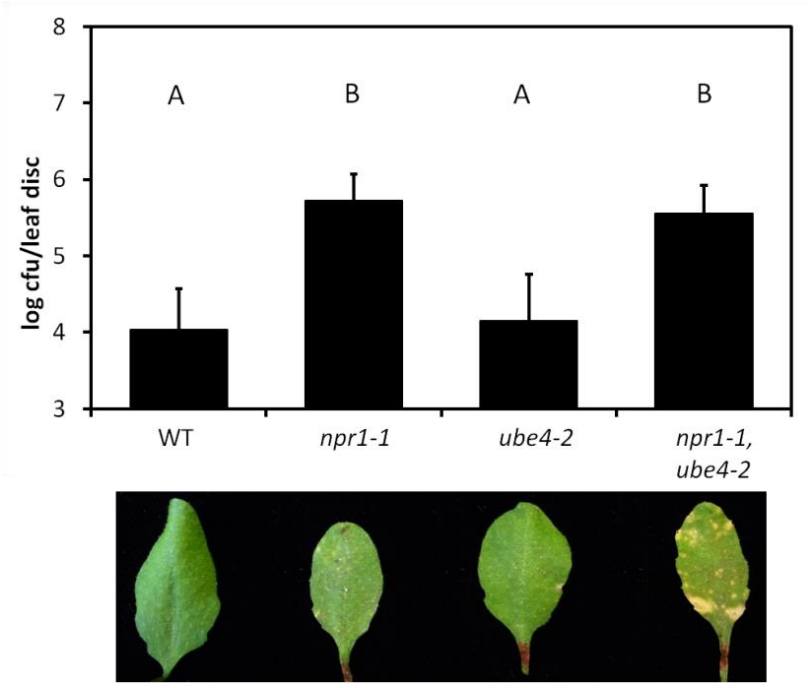

Figure 3.8: UBE4 requires the SA master regulator NPR1 to maintain basal immune defences.

Enhanced disease susceptibility test: Top: Quantification of pathogen levels within infected leaves. Bottom: Morphology of infected leaves. 3-week old adult plants were inoculated with 5 x 10^5 colony forming units/ml *Psm* ES4326 into the abaxial surface of a leaf via syringe infiltration. Leaf discs were collected 5-days post inoculation, bacteria was extracted and serial diluted before colony counting on selective agar plates. Error bars indicate 95 % confidence limits (n = 8). Letters indicate significantly different samples (Tukey-Kramer ANOVA test, α = 0.05, n = 8).

Taken together the results of the genetic crosses to *sid2-2* and *npr1-1* indicate that UBE4 has a functionally relevant position within SA-mediated immune responses that are NPR1 dependent.

3.2.5. UBE4 localizes to both the nucleus and cytoplasm

Because the effect of *ube4* on plant defence expression was NPR1 dependent (Figures 3.7 & 3.8), we investigated if UBE4 protein localizes to the same cellular compartments as NPR1. NPR1 is present in both the nucleus and the cytoplasm. In the cytoplasm NPR1 is found mainly as a large, redox-sensitive oligomer with small amounts moving into the nucleus to maintain basal gene expression. Upon activation of SA signalling, a redox change leads to a greater amount of NPR1 becoming monomerized, which enter the nucleus and can activate gene transcription (Tada et al., 2008, Spoel et al., 2009, Kinkema et al., 2000, Mou et al., 2003). UBE4 was fused to Yellow Fluorescent Protein (YFP) and placed under control of the constitutive cauliflower mosaic virus 35S promoter to create the *35S::YFP-UBE4* construct, which was transformed and expressed in protoplasts. As can been seen in figure 3.9A, YFP-UBE4 localized to both the nucleus and the cytoplasm, suggesting it could interact with NPR1. Protoplasts transformed with YFP-UBE4 were also examined via Western blot (figure 3B), an observed band of YFP-UBE4 confirms the fluorescence seen in figure 3A was not due to cleaved YFP.

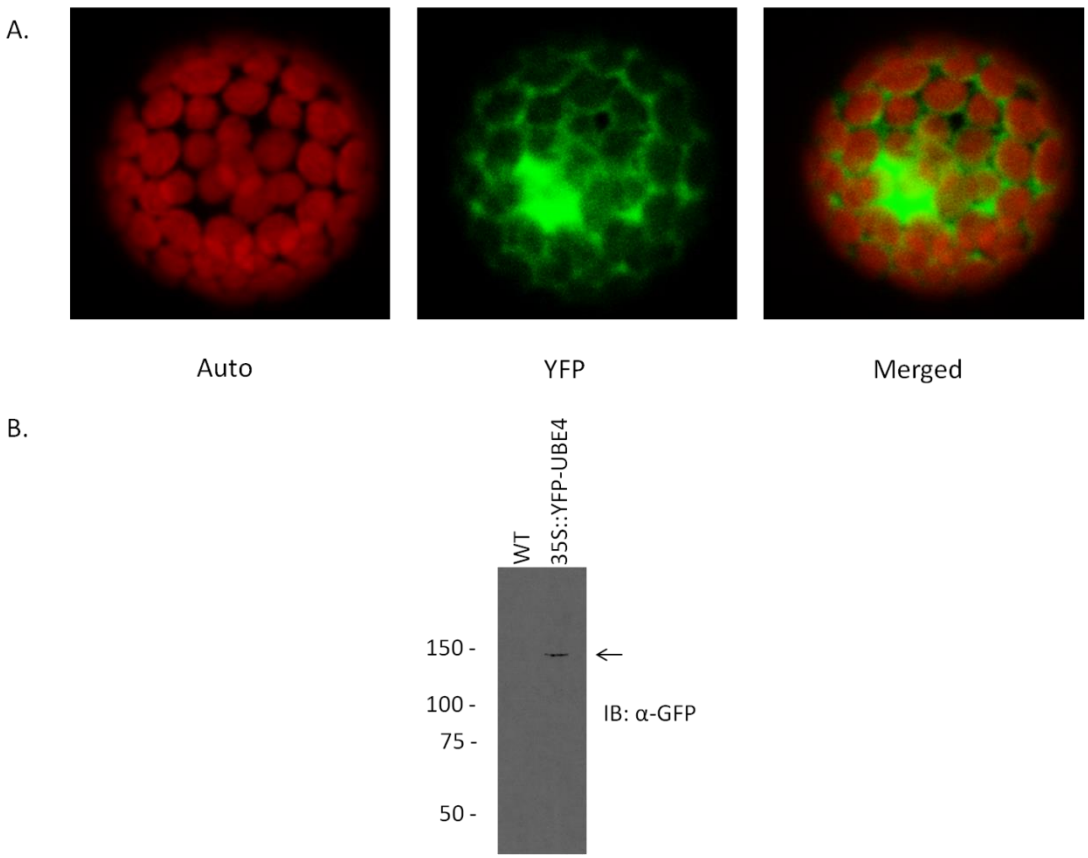

Figure 3.9: YFP-UBE4 is localised to the nucleus and cytoplasm.

A. *35S::YFP-UBE4* was transformed into protoplasts and subcellular localization analysed by confocal microscopy. Left: Auto-fluorescence of protoplasts. Middle: 35S::YFP-UBE4. Right: Merged image. **B.** Western blot of protoplasts transformed with 35S::YFP-UBE4. Arrow indicates YFP-UBE4.

3.2.6. UBE4 is a ubiquitin ligase

As UBE4 is a putative homolog of the yeast E4 ligase UFD2 (Figure 3.1) (Huang et al., 2014), we set out to discover if the *Arabidopsis* UBE4 also functions as a ubiquitin ligase. Ubiquitinated proteins were purified from wild type and mutant *ube4-2* plants using Tandem Ubiquitin Binding Entities (TUBE) (Hjerpe et al., 2009). TUBE proteins consist of four ubiquitin binding domains linked together by a flexible linker and attached to a His or GST tag. Although TUBE proteins can bind single ubiquitin moieties they have higher affinity for polyubiquitin chains, allowing easy purification of polyubiquitinated proteins (Hjerpe et al., 2009). Seedlings were immersed for six hours in either water, 0.5 mM SA, or a combination of 0.5 mM SA and 100 µM MG132, a proteasome inhibitor. Protein was extracted and analysed by SDS-PAGE and Western blotting. When probed with an anti-ubiquitin antibody, water- and SA-treated wild type plants showed a smear of polyubiquitinated proteins of different molecular weights (Figure 3.10). When the proteasome was inhibited by inclusion of MG132, the level of polyubiquitinated proteins strongly increased. In *ube4-2* plants the level of polyubiquitination was similar to that of wild type (Figure 3.10). We then probed the blot with an antibody against RPN10, which acts as a ubiquitin receptor for the proteasome, but RPN10 itself is heavily ubiquitinated by a large variety of different ubiquitin ligases (Uchiki et al., 2009, Kim and Goldberg, 2012) and thus it was used here as a model substrate for polyubiquitination. Indeed, polyubiquitination of RPN10 was readily detected as a wide variety of high molecular weight bands (Figure 3.10). In SA-treated wild type plants, the intensities of polyubiquitinated RPN10 bands increased after addition of SA and especially in presence of MG132. Strikingly, *ube4-2* mutants accumulated less polyubiquitinated RPN10 than wild type and this level was largely unresponsive to the addition of SA. After MG132 addition the amounts of polyubiquitinated RPN10 were too high to make a reliable comparison to

wild type. These data demonstrate that UBE4 enhances levels of poly-ubiquitination on RPN10, suggesting it may indeed function as an E4 ubiquitin ligase in *Arabidopsis*.

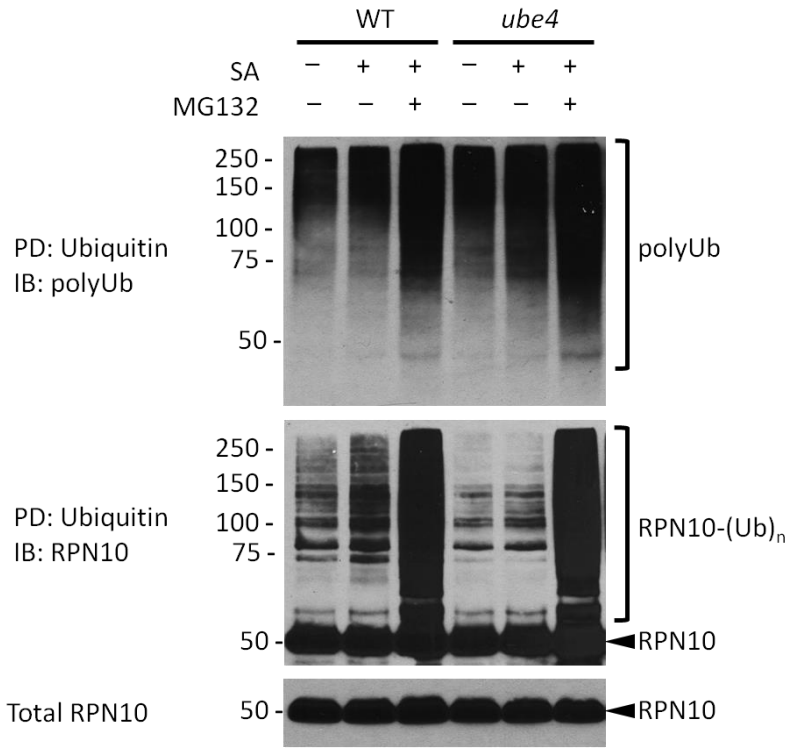

Figure 3.10: UBE4 is a ubiquitin ligase that acts upon the ubiquitination model substrate RPN10.

Approximately 75 two-week old seedlings grown on MS-agar plates were immersed for 6 hours in solutions containing vehicle (DMSO), 0.5 mM SA or 100 μM MG132 as indicated before protein extraction in the presence of His-tagged TUBE protein to capture ubiquitinated proteins. His-TUBE and associated polyubiquitinated proteins were pulled down (PD) on His-Pure cobalt beads. Eluate was analysed by SDS-PAGE and probed by immunoblotting (IB) with α-ubiquitin and α-RPN10 antibodies. A sample of crude extract before purification on His-Pure cobalt beads was used to provide total RPN10.

3.2.7. Mutation of *UBE4* stabilises NPR1 coactivator

As UBE4 can act as a ubiquitin ligase (Figure 3.10) and influences NPR1-dependent gene expression and immunity (Figures 3.3 – 3.8), it was hypothesized that UBE4 may alter the stability of NPR1 protein. To investigate this possibility NPR1 fused to green fluorescent protein (NPR1-GFP) was constitutively expressed in *npr1-1* single and *ube4-2 npr1-1* double mutants. The resulting *35S::NPR1-GFP* (*npr1-1*) and *35S::NPR1-GFP* (*ube4-2 npr1-1*) lines were subjected to a cycloheximide chase in which protein synthesis is blocked by the presence of the ribosomal inhibitor cycloheximide, allowing the rate of protein degradation to be measured. Seedlings were placed in solutions containing 0.5 mM SA and 100 µM cycloheximide and samples were taken at various time-points. In wild type plants NPR1-GFP was almost completely degraded by 6 hours and undetectable by 8 hours (Figure 3.11). By contrast, in *ube4-2* plants NPR1-GFP was more abundant than in the wild type at 6 hours and still detectable at 8 hours. This result suggests that in *ube4-2* plants NPR1 is degraded at a slower rate than in the wild type.

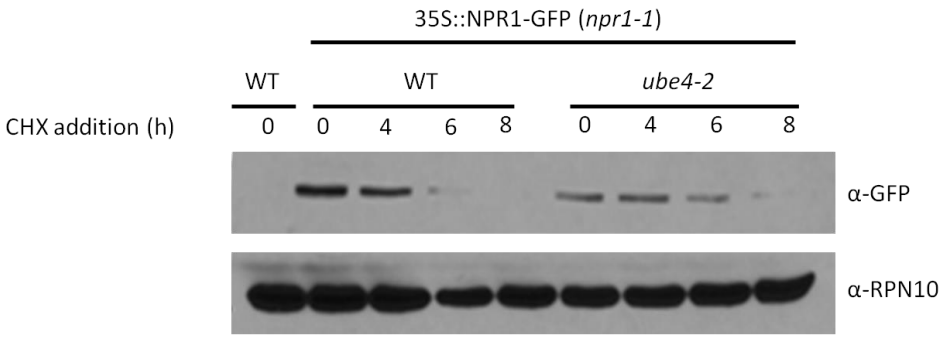

Figure 3.11: Knock-out of *UBE4* leads to stabilisation of NPR1-GFP.

Approximately 75 two-week old seedlings grown on MS-agar plates were immersed in a 0.5 mM SA and 100 µM cycloheximide solution and samples collected at indicated time-points. Protein extracts were analysed by SDS-PAGE and probed with antibodies against GFP and RPN10, the latter of which functioned as a loading control.

3.2.8. UBE4 functions as a ubiquitin ligase for NPR1

Because UBE4 acts as a ubiquitin ligase on at least one substrate (RPN10) (Figure 3.10) and can stabilise NPR1 (Figure 3.11), we explored the hypothesis that UBE4 could also act as a ubiquitin ligase of NPR1. Therefore, *35S::NPR1-GFP* (*npr1-1*) and *35S::NPR1-GFP* (*ube4-2 npr1-1*) seedlings were treated for 4 hours with water or 0.5 mM SA and subsequently 40 µM MG132 was added for an additional 2 hours. Samples were collected and immunoprecipitation performed using an antibody against GFP followed by SDS-PAGE and western blotting for both ubiquitin and GFP (Figure 3.12). This method readily detected high molecular weight, polyubiquitinated NPR1-GFP but also revealed a faster migrating, potentially monoubiquitinated form of NPR1-GFP (Figure 3.12). However, follow-on experiments indicated that the anti-ubiquitin antibody sporadically detected the large amounts of immunoprecipitated unmodified NPR1-GFP (data not shown) in this type of experiment. Regardless of this, SA stimulated polyubiquitination of NPR1-GFP in wild type plants, while polyubiquitinated NPR1-GFP levels in *ube4-2* mutants were unresponsive to SA. These data indicate UBE4 may be required to elongate ubiquitin chains on NPR1-GFP in a SA-dependent manner.

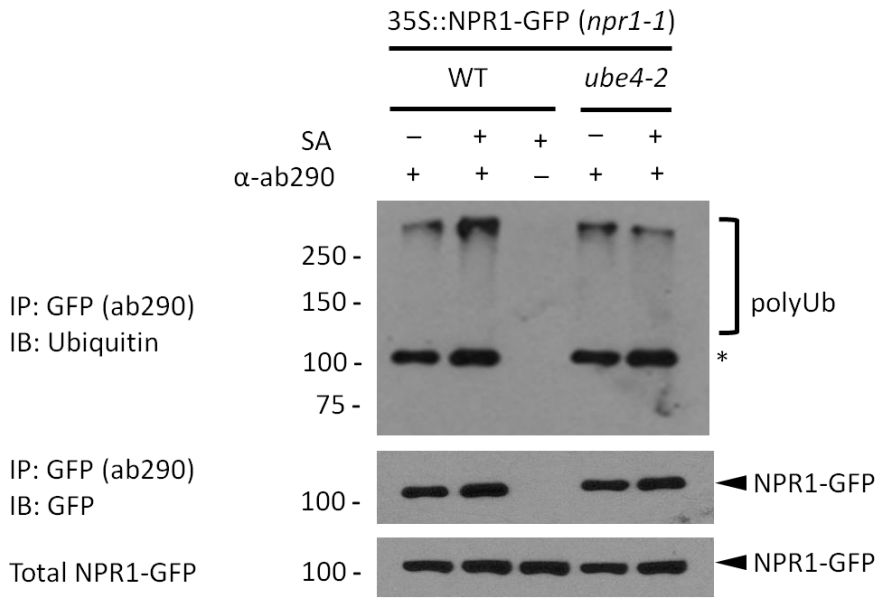

Figure 3.12: Mutant *ube4*-2 plants have decreased levels of polyubiquitinated NPR1-GFP.

Approximately 75 two-week old seedlings were immersed for 4 hours in water or 0.5 mM SA before addition of 40 μM MG132 for an additional 2 hours. Immunoprecipitation (IP) was performed on protein extract in presence or absence of the α-ab290 (anti-GFP) antibody. Eluate was analysed by SDS-PAGE and immunoblotting (IB) with ubiquitin and GFP antibodies. Asterisk indicates non-ubiquitinated NPR1-GFP.

To confirm the results seen in the IP using the GFP antibody, a reverse pulldown was performed using the GST-tagged Tandem Ubiquitin Binding Enitity (TUBE) (Hjerpe et al., 2009). TUBE proteins bind ubiquitinated substrates with high affinity allowing pulldowns to be performed and proteins of interest probed for with antibodies. The results using the TUBE method mirror those of the IP in figure 3.12. NPR1-GFP can be detected as a smear indicating ubiquitin chains of various lengths are present and their intensities increased after the addition of SA (Figure 3.13). Note that unmodified NPR1-GFP was also pulled down by the TUBE method, indicating NPR1 interacts with its own polyubiquitinated form or other polyubiquitinated proteins. Although mock-treated *ube4-2* and wild type plants did not differ, SA treatment of *ube4* mutants did not induce an increase in the amount of

polyubiquitinated NPR1-GFP. These data show that correct polyubiquitination of NPR1-GFP requires the E4 ligase UBE4.

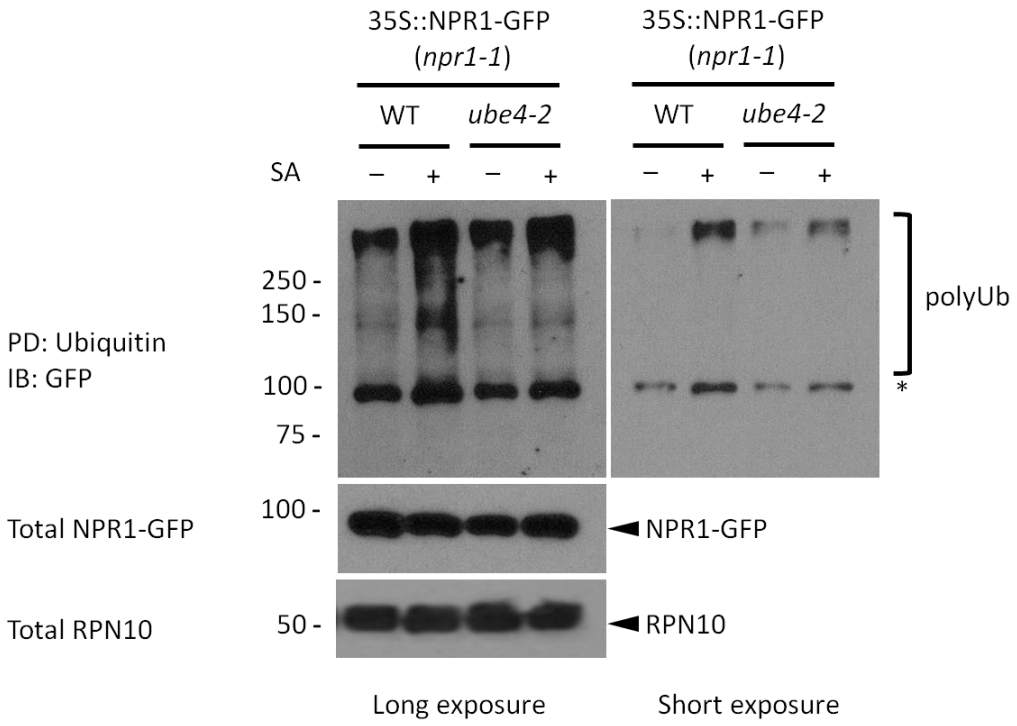

Figure 3.13: Pull-down of ubiquitinated protein using TUBE and probing with α-GFP confirms that in *ube4-2* NPR1-GFP has lower poly-ubiquitination levels.

Approximately 75 14-day old seedlings were immersed in 0.5 mM SA (+) for 4 hours or water (-) before addition of 40 μM MG132 for 2 hours. Pull down (PD) was performed on protein extraction in the presence of GST-tagged TUBE protein to capture ubiquitinated proteins. GST-TUBE and associated polyubiquitinated proteins were pulled down (PD) on glutathione beads. Eluate was analysed by SDS-PAGE and immunoblotted (IB) with ubiquitin, GFP and RPN10 antibodies. Asterisk indicates non-ubiquitinated NPR1-GFP.

3.2.9. UBE4 suppresses transcriptional activity of NPR1

Mutation of the E3 ubiquitin ligase CUL3 results in untimely activation of plant immune responses (Spoel et al., 2009). To investigate if UBE4 plays a similar role in plant immunity, we examined the expression of SA-responsive immune genes in absence of pathogen challenge. Adult plants were each placed in a 5 ml well containing 0.5 mM SA or water (Mock) for 24 hours. Two leaves from each plant were collected and used for qPCR. Treatment with SA induced expression of the marker genes *PR-1*, *WRKY18*, *WRKY38* and *WRKY62* in both wild type and *ube4-2* plants. However, compared to the wild type, mutant *ube4-2* plants exhibited far higher levels of immune gene expression (Figure 3.14). This indicates that UBE4 may play a role in regulating defence gene expression.

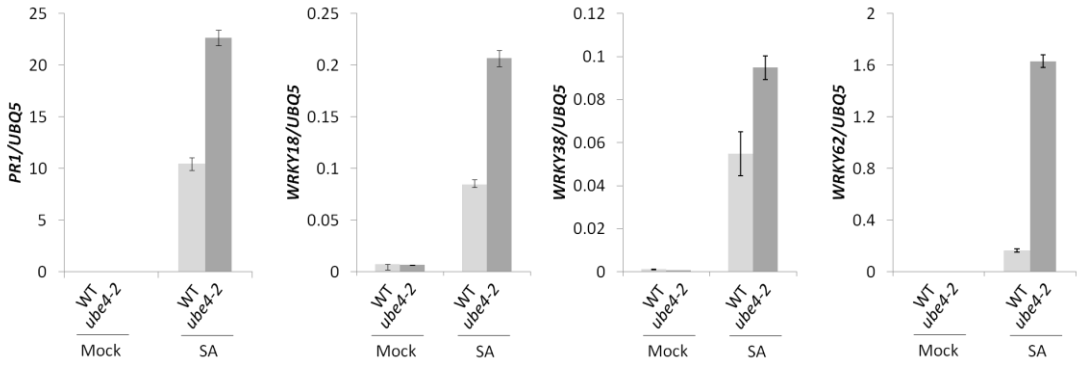

Figure 3.14: Increased SA-induced defence gene expression is observed in *ube4-2.*

Adult wild type and *ube4-2* plants were placed in 5 ml wells containing 0.5 mM SA or water (Mock) for 24 hours. Two leaves from 3 plants were collected, pooled and used for qPCR with gene specific primers against SA-responsive *PR-1*, *WRKY18*, *WRKY38*, and WRKY62. Expression was normalized against constitutively expressed ubiquitin 5 (UBQ5). n = 3. Error bars indicate standard error from 3 technical replicates.

3.2.10. The ubiquitin ligases CUL3 and UBE4 have opposing activities in SA-dependent gene expression

A previous report indicated that ubiquitination by a CUL3-based E3 ligase and subsequent proteasome-mediated turnover of NPR1 were required for full activation of its target genes (Spoel et al., 2009). Here we showed, however, stabilization of NPR1 in the *ube4-2* background is associated with increased transcription of its target genes. To further investigate this discrepancy we compared SA-induced NPR1 target gene expression in *cul3a cul3b* and *ube4-2* mutants side by side. Seedlings were placed into 0.5 mM SA or water for 6 h and then analysed by qPCR. As seen in figure 3.15, addition of SA to wild type plants induced high levels of transcription of all NPR1 target genes tested, except *PR-5*. Mutant *npr1* plants lacked the ability to respond to SA (Wang et al., 2006) and marker transcription levels were indeed very low. In *cul3* mutants transcription levels increased but remained lower than that of wild type. In contrast, *ube4-2* mutants displayed increased gene transcription compared to wild type for nearly all of the genes tested (Figure 3.15). These results suggest that while the E4 ligase UBE4 and the E3 ligase CUL3 both stabilize NPR1 protein levels, they have opposing effects on NPR1 target gene transcription.

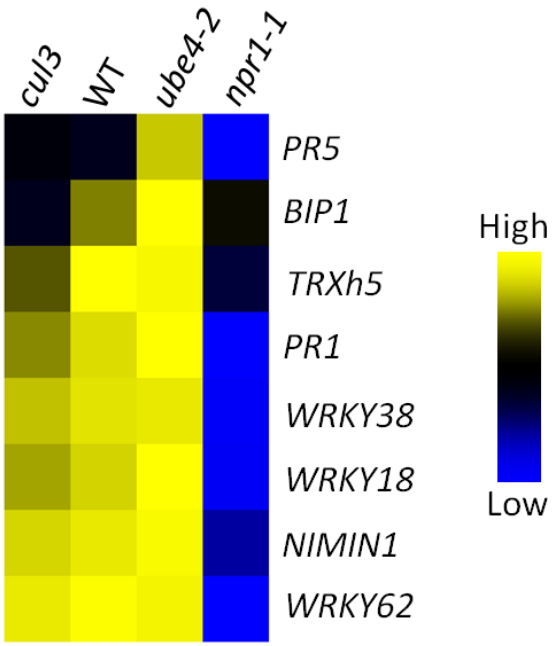

Figure 3.15: Mutation of the E4 ligase UBE4 has the opposite effect on NPR1 target gene transcription as the E3 ligase mutant *cul3*.

Seedlings were placed into 0.5 mM SA or water for 6 hours and then analysed by qPCR for the expression of SA-responsive NPR1 target genes. All expression levels were normalised to UBQ5 and converted to a heat map.

3.2.11. The *ube4-2* mutant uncouples NPR1 coactivator activity from proteasome-mediated turnover

Knockout of UBE4 leads to reduced polyubiquitination with increased SA-dependent gene expression, hinting that in *ube4-2* plants NPR1 activity has been uncoupled from proteasome-mediated degradation. To test this hypothesis, seedlings were immersed in 0.5 mM SA for 4 hours and subsequently supplemented with various concentrations of MG132 for another 2 hours. Addition of MG132 led to strong concentration-dependent decreases in SA-induced expression of *PR1* and *WRKY18* in wild type plants. By contrast, in *ube4-2* mutants *PR1* expression remained high in presence of 40 and 80 µM MG132 and suppression of transcription only became apparent with 100 µM MG132. Moreover, WRKY18 expression in *ube4-2* mutants was not decreased by any of the applied MG132 concentrations (Figure 3.16). Hence, knockout of *UBE4* leads to a decoupling of NPR1-dependent transcription from proteasome activity, suggesting that initial ubiquitination of NPR1 is necessary for it activity, but extended polyubiquitination and proteasome-mediated degradation serve to limit that activity.

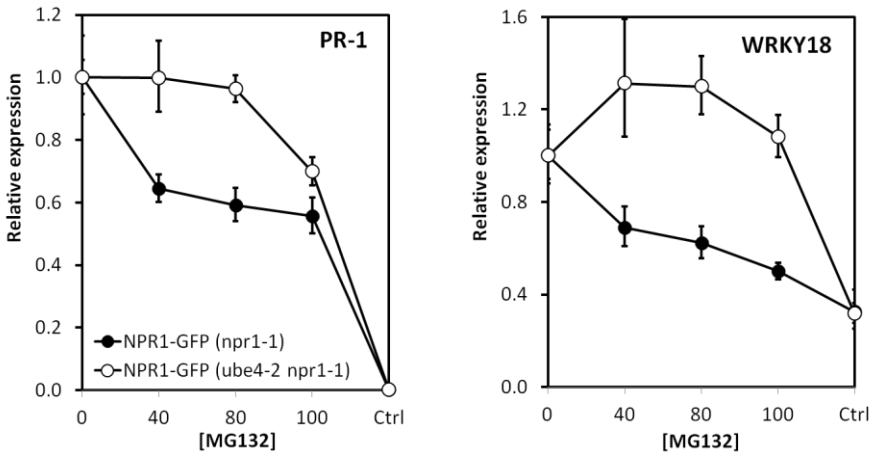

Figure 3.16: The *ube4-2* mutant uncouples NPR1-dependent gene transcription from proteasome activity.

Approximately 75 two-week old seedlings were immersed in 0.5 mM SA for 4 hours or water (Ctrl) before addition of MG132 at various concentrations (in µM) for an additional 2 hours. RNA was extracted and used for qPCR with gene specific primers against SA-responsive *PR-1* and *WRKY18*. Expression was first normalized against constitutively expressed ubiquitin 5 (UBQ5) and then expressed relative to plants that were treated with SA alone (i.e. 0 µM MG132). Ctrl = expression level in water-treated plants. Bars indicate standard error from 3 technical replicates.

3.3. Discussion

In *Arabidopsis* the immune response to biotrophic pathogens is regulated by the SA-activated transcription coactivator NPR1. Previous work has shown that in order to correctly orchestrate changes in gene expression induced by SA, NPR1 requires sumoylation by SUMO3 to allow it to switch from binding the WRKY70 repressor to the coactivator TGA3 (Saleh et al., 2015). The transcriptionally competent NPR1 is subject to proteasome-mediated turnover signalled by the ubiquitin ligase CUL3, after a round of transcription has been initiated (Spoel et al., 2009). However, it remains largely unknown if ubiquitination of NPR1 affects its intrinsic transcriptional activity. Here we investigated this by analysing the role of the E4 ubiquitin ligase, UBE4, in controlling NPR1 activity. We found that mutation of UBE4 decreased the levels of polyubiquitination, leading to stabilisation of NPR1. Contrary to mutation of CUL3, however, *ube4* mutants displayed increased gene transcription that was independent of proteasome-mediated destruction of NPR1. We propose that lower order ubiquitin chains increase transcriptional activity of NPR1 and that proteasome-mediated degradation serves to limit that activity, providing rapid and accurate control over transcriptional responses to SA.

Using sequence alignment and homology modelling, we identified *Arabidopsis* UBE4 as a homologue of the yeast E4 ligase UFD2 (Figure 3.1)(Huang et al., 2014). Knockout of *UBE4* led to increased basal immune gene expression and immunity (Figures 3.3, 3.5 & 3.7), indicating that UBE4 is required for timely gene expression. Genetic dissection of the SA response pathway indicated that constitutive gene expression and immunity in *ube4-2* mutants required the presence of SA and specifically the coactivator NPR1 (Figures 3.5 - 3.8). Previous reports indicate that NPR1 is continuously targeted to the proteasome by a CUL3-based E3 ligase that utilizes the adaptors NPR3 and NPR4 (*i.e.* $CUL3^{NPR4}$ and $CUL3^{NPR3}$)

(Spoel et al., 2009, Fu et al., 2012). In the resting state NPR1 exists as an oligomer within the cytoplasm, but small amounts of the NPR1 monomer are believed to escape and enter the nucleus where they are degraded via $CUL3^{NPR4}$ to prevent spurious activation of defence responses (Fu et al., 2012). In mutant *cul3* and *npr3 npr4* plants an increase in basal immune defences similar to that in *ube4-2* has been reported (Spoel et al., 2009, Fu et al., 2012). The upregulated expression in both mutants of UBE4 and $CUL3^{NPR3/4}$ was abolished by crossing to the *npr1-1* background (Figure 3.15) (Spoel et al., 2009, Fu et al., 2012). These data suggest that and $CUL3^{NPR4}$ and UBE4 may both act on NPR1, and perhaps in sequence, to prevent untimely autoimmune responses.

Mutation of CUL3 or NPR1's degron motif led to a reduction of its polyubiquitination accompanied by NPR1 protein stabilisation (Spoel et al., 2009). Our results suggest that UBE4 also exhibits ubiquitin ligase activity on NPR1 (Figures 3.12 & 3.13). Thus, the reduction in polyubiquitinated NPR1 in *ube4-2* mutants is likely responsible for its stabilisation (Figure 3.11). In yeast, UFD2 acts as an E4 ligase, increasing the length of chains on substrates that have already been partially ubiquitinated by the action of E3 ligases (Koegl et al., 1999, Saeki et al., 2004). Due to the high degree of homology between ScUFD2 and AtUBE4 (Figure 3.1 & Supplemental 3.1), the reduction of detectable NPR1-GFP polyubiquitination in *ube4-2* is likely due to reduced chain length. In *Arabidopsis*, UBE4 has been proposed to act on the pathogen receptor proteins suppressor of *npr1-1,* constitutive (SNC1) and resistant to *P.syringae* 2 (RPS2). It was observed that UBE4 enhanced degradation of the receptor proteins in the presence of the E3 ligase $CUL1^{CPR1}$, but UBE4 had no effect on the degradation of SNC1 and RPS2 in absence of the Constitutive expressor of PR genes 1 (CPR1) F-box protein, indicating that UBE4 acts as an E4 ligase within this ubiquitination pathway. Mutation of *UBE4* led to increased stabilisation of the receptor proteins, but a double mutant of *ube4* and *snc1* failed to completely suppress the

increased basal *PR-1* and *PR-2* expression seen in the *ube4* single mutant (Huang et al., 2014). The authors proposed that UBE4 may act on other receptor proteins that contribute alongside SNC1 to the *ube4* mutant phenotype of enhanced immune gene expression. However, as we showed here that UBE4 acts on the master regulator NPR1, it is likely that the incomplete suppression is probably due to mis-regulation of NPR1. Furthermore, Huang et al. (2014) found that mutation of SNC1 did not prevent the disease resistance phenotype that *ube4* mutant plants displayed against *Psm* ES4326. Data presented in this chapter now clearly indicate that resistance against *Psm* ES4326 infection in *ube4* mutants is due to enhanced transcriptional activity of NPR1 coactivator. Hence, UBE4 has been shown to act in cooperation with two different members of the CUL ubiquitin ligase family; the $CUL1^{CPR1}$ ligase (Huang et al., 2014) and $CUL3^{NPR3/4}$ ligase (this chapter). Therefore, it is possible that UBE4 acts as a general ubiquitination enhancement factor for CUL-based ubiquitin ligases.

Although UBE4 likely acts as a ubiquitin ligase for NPR1, we have not yet been able to co-immunoprecipitate NPR1 and UBE4 (data not shown). This may be due to the interaction being too transient to catch or the interaction requiring an unstably associated adaptor protein. UBE4 has been found to have a direct interaction with SNC1 but not RPS2, leading the authors to speculate that an adaptor was required to alter UBE4 specifity (Huang et al., 2014). Indeed if UBE4 acts in a generalist manner i.e. it enhances ubiquitination on many separate target substrates mediated by a variety of E3s ligases, an adaptor is likely required to increase the specificity of UBE4 interaction with a particular substrate.

UBE4 was found to localize to both the cytoplasm and the nucleus (Figure 3.9) (Huang et al., 2014), consistent with its reported interactions with SNC1 and RPS2 immune receptors (Huang et al., 2014) and with its predicted interaction with nuclear NPR1 as

reported in this Chapter (Figures. 3.12 & 3.13). A key point with regards to the interaction with NPR1 is where in the nucleus it occurs. To conclusively prove UBE4 alters gene transcription via ubiquitination of transcriptional active NPR1, interaction between UBE4 and NPR1 on the chromatin via chromatin immunoprecipitation is required.

The key difference between NPR1 protein stabilisation caused by mutation of *CUL3* or *UBE4* is that the *cul3* mutant showed reduced NPR1-dependent transcription, whereas *ube4-2* mutants exhibited increased levels of transcription after application of SA (Figure 3.14 & 3.15). This discrepancy strongly implies a scenario in which initial CUL3-mediated ubiquitination of NPR1 is necessary for this coactivator to attain full transcriptional activity, while subsequent ubiquitin chain extension by UBE4 inactivates NPR1 by both stoichiometric inhibition and targeting to the proteasome.

Previous work within plants exploring ubiquitination of TFs has focused on polyubiquitination being used to regulate protein degradation (Furniss and Spoel, 2015, Trujillo and Shirasu, 2010, Stone, 2014, Vierstra, 2009). However, in other eukaryotic systems ubiquitination of TFs directly alters their behaviour. This notion was first explored in yeast. Transcription induced by an artificial activator consisting of the yeast VP16 transactivation domain and the bacterial LexA DNA binding protein (LexA-VP16), was shown to require ubiquitination and degradation mediated by the F-box protein methionine requiring 30 (MET30). Strikingly, when ubiquitin was fused in-frame to LexA-VP16, the requirement for MET30 was completely bypassed (Salghetti et al., 2001), suggesting that ubiquitination has dual functions to both activate and destroy transcription activators. Subsequently, additional studies indicated roles for monoubiquitination in transcription activation (Bres et al., 2003, Greer et al., 2003, Burgdorf et al., 2004). Monoubiquitination does not usually signal for proteasome-mediated degradation, for which approximately

four or more K48-linked ubiquitins are required (Thrower et al., 2000). Instead it was reported that promoter occupancy of the yeast prototypical transcription activator, Galactose metabolism 4 (GAL4), was stabilized by monoubiquitination (Ferdous et al., 2007, Archer et al., 2008b). Interestingly, unmodified GAL4 was destabilized by ATPase activity of the proteasome 19S regulatory particle (RP), preventing transcription activation. Monoubiquitination limited the lifetime of physical interactions between the GAL4 activation domain and RP subunits (Archer et al., 2008a). This type of regulatory system likely extends to many other eukaryotes, as interactions between tumor suppressor protein p53, a transcription activator in mammalian cells, and its target promoters were also destabilized by the RP ATPases (Kim et al., 2009).

While monoubiquitination may play a regulatory role, processive ubiquitin chain elongation subsequently leads to activator turnover (Kodadek et al., 2006). This processive mono-to-polyubiquitination switch was explored in particular detail for the human Steroid Receptor Coactivator-3 (SRC-3). SRC-3 is an important developmental transcription coactivator, whose uncontrolled expression can lead to oncogenesis. SRC-3 was found to be subject to phosphorylation-dependent polyubiquitination by the ligase Fbw7α, resulting in its transcription-coupled degradation. However, SRC-3 was also multi(mono)-ubiquitinated by Fbw7α, which enhanced its transcriptional activity. Hence, it was proposed that biphasic, processive ubiquitination (*i.e.* transitioning from mono- to polyubiquitination) generates a timer for the functional lifetime of SRC-3 (Wu et al., 2007). Here we have shown that processive ubiquitination of the transcription coactivator NPR1 is regulated by UBE4 acting in the capacity of an E4 ligase. We propose that mono- or lower order ubiquitination produces a highly transcriptionally active NPR1, which operates within a window of opportunity that is limited by UBE4-mediated polyubiquitination and subsequent degradation by the proteasome. Indeed, chemical inhibition of the proteasome through

application of MG132 leads to a reduction of SA-induced gene expression in wild type, while in *ube4-2,* addition of MG132 had little effect (Figure 3.16), confirming turnover of polyubiquitinated NPR1 is normally required for timely and accurate gene expression levels. We therefore conclude that the increased transcriptional activity of NPR1 by ubiquitination and its subsequent destruction provides a rapid and reliable way to keep tight regulation on both the level and length of SA-dependent gene expression.

Chapter 4: Discovery and characterisation of a novel ubiquitin ligase crucial to SA-dependent immunity

4.1. Introduction

UBE4 is the only E4 ligase identified within *Arabidopsis* so far and is homologous to the yeast E4 ligase, UFD2 (Chapter 3) (Huang et al., 2014). However, another well-defined E4 ligase in yeast is the HECT domain-containing HUL5 protein. HUL5 was discovered as a proteasome accessory protein through a salt labile interaction (Leggett et al., 2002) and acts to increase the ubiquitin chain length on ubiquitinated substrates (Crosas et al., 2006). Deletion of HUL5 leads to substrate stabilisation and is associated with a reduction in substrate polyubiquitination. HUL5 has been shown to act in direct opposition to the deubiquitinase UBP6, the deletion of which has the opposite effect of increasing turnover of polyubiquitinated substrates (Crosas et al., 2006, Leggett et al., 2002). HUL5 has also been shown to be responsible for the generation of free ubiquitin chains, a function that it shares with another HECT domain-containing E4 ligase, ubiquitin fusion degradation 4 (UFD4) (Braten et al., 2012). HUL5 produces ubiquitin chains in response to stress conditions (Braten et al., 2012), such as heat-shock in which HUL5 is involved in the degradation of mis-folded proteins (Fang et al., 2011), whereas UFD4 regulates basal synthesis of free ubiquitin chains (Braten et al., 2012). In *Arabidopsis* there are seven HECT domain-containing proteins (El Refy et al., 2004), all of which are part of the ubiquitin protein ligase (UPL) family. UPL3 is similar in homology to the yeast protein UFD4; both proteins contain Armadillo (Arm) domains used for protein-protein interactions (El Refy et al., 2004, Downes et al., 2003). UPL3 has been implicated in the control of trichome formation, as knockout of UPL3 leads to increased trichome branching due to excessive endoreduplication (Downes et al., 2003, El Refy et al., 2004). It was subsequently

discovered that UPL3 is responsible for the degradation of two bHLH transcription cofactors, glabrous 3 (GL3) and enhancerof GL3 (EGL3), involved in the promotion of trichome development and flavonoid synthesis (Patra et al., 2013). UPL3 interacts with GL3 and EGL3 by physically associating its armadillo domains to the C-terminal domains of these transcription cofactors. Knockout of UPL3 leads to stabilisation of both GL3 and EGL3, however, GL3 has been implicated in binding the UPL3 promoter and in a *gl3* mutant *UPL3* transcription was reduced; these data indicate the presence of a regulatory feedback loop (Patra et al., 2013). Another UPL protein, UPL5, has been implicated in leaf senescence. UPL5 acts as a repressor of a key senescence promoter, WRKY53, by targeting it for ubiquitination and proteasome-mediated degradation. Accordingly, mutation of UPL5 led to stabilisation of WRKY53 and early senescence, in a manner similar to WRKY53 over-expressers. In wild-type plants WRKY53 and UPL5 act antagonistically. UPL5 levels remain stable throughout development of the plant except during bolting when WRKY53 drives senescence and UPL5 levels decrease (Miao and Zentgraf, 2010). WRKY53 has also been shown to be a direct target of NPR1, and acts as a positive regulator of SA-dependent immunity alongside WRKY70 which is also involved in leaf senescence as a negative regulator (Wang et al., 2006, Miao and Zentgraf, 2010, Zentgraf et al., 2010) Thus, UPL3 and UPL5 both act as ubiquitin ligases of transcription (co)factors and mutants showed defects in gene regulation, making the UPL family excellent candidates for functioning as putative E4 ligases in the plant immune response.

In this chapter we investigated the role of several UPL genes and their ability to regulate total cellular ubiquitination levels and polyubiquitination of a model substrate. As we are ultimately interested in discovering ubiquitin ligases involved in the regulation of immune transcription we tested UPL mutants for SA-induced gene expression and disease resistance. We found that reduced levels of ubiquitination in mutants of the closely related

UPL3 and UPL4 proteins correlated with severe defects in SA-mediated gene expression and disease resistance, indicating these ubiquitin ligases play essential roles in orchestrating the plant immune response.

4.2. Results

4.2.1. Ubiquitin Protein Ligases are putative homologs of the yeast E4 ligase HUL5

Using the ScHUL5 protein sequence putative *Arabidopsis* homologues were identified via a BLASTP search, yielding the seven UPL proteins (Supplemental 4.2). All seven UPL genes contain the HECT domain (Figure 4B). Due to the availability of T-DNA insertion mutants we investigated the UPL3, UPL4, UPL6 and UPL7 genes, which fall into two pairs of paralogues (UPL3/UPL4 and UPL6/UPL7, Figure 4A). As well as a HECT domain, UPL3 and UPL4 both feature Armadillo repeats similar to those found on the nuclear pore protein importin-α (Figures 4A & 4C) (Downes et al., 2003), whereas UPL6 and UPL7 feature isoleucine-glutamine (IQ) calmodulin binding domains (Mitchell et al., 2015) and reported transmembrane domains (Figure 4.1A) (Downes et al., 2003).

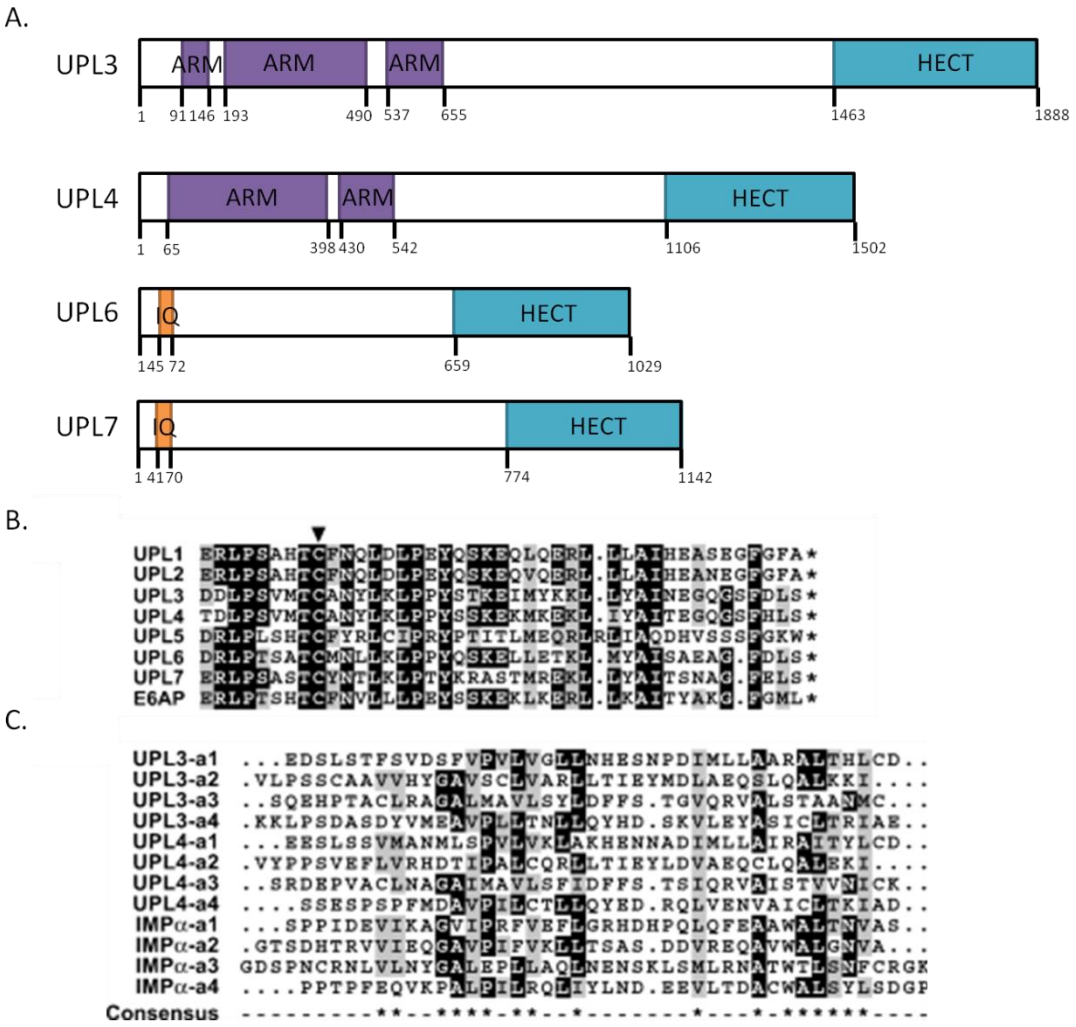

Figure 4.1: Domain structure of UPL proteins

A. Domain structure drawn from results generated by InterPro supplied with the protein amino acid sequence from TAIR 10. Numbers indicate amino acid position. ARM: Armadillo domains. HECT: Homologus to E6-AP Carboxyl Terminus Domain. IQ: Isoleucine-Glutamine motif. **B**. Alignment of UPL HECT domains to the E6-AP Carboxyl Terminus Domain. Arrow indicates active-site cysteine. Asterisk indicates the end of the sequence. E6-AP Carboxyl Terminus Domain **C.** Alignment of UPL3 and UPL4 Armadillo domains to importin-α. Asterisks indicates residues conserved in diverse eukaryotic organisms. Figures 4.1B & C taken from Downes et al (2003).

4.2.2. Phenotypes of *upl* mutants

Two T-DNA insertion mutants were acquired from the SALK institute for each gene of interest (Alonso et al., 2003) and genotyped to confirm presence of the T-DNA in the correct genomic location. All alleles contained the correct T-DNA insertions except for *upl3-2* plants which were found to be wild type (data not shown). RT-PCR was performed to confirm if the T-DNA knocked out expression of the gene of interest. All alleles assayed were knockouts, except for *upl6-2* which was a knock-down (Figure 4.2) Knockout of *UPL4*, *UPL6*, *UPL7*, including the *upl6 upl7* double mutant, resulted in wild type growth and showed no obvious phenotypic effects (Figure 4.3). UPL3 has been previously described as KAKTUS and is involved in trichome development; *upl3* trichomes usually have 5-6 branches instead of the normal 3 (Downes et al., 2003), which we also observed (data not shown). As *upl3-1* was a knockout (Figure 4.2) and carried the same phenotype as previously reported, we carried on our investigation with the single mutant allele. Compared to parent single mutants, *upl3 upl4* double mutant offspring showed reduced growth (Figure 4.3), senesced earlier and produce considerably less seed (data not shown), indicating UPL3 and UPL4 may function redundantly.

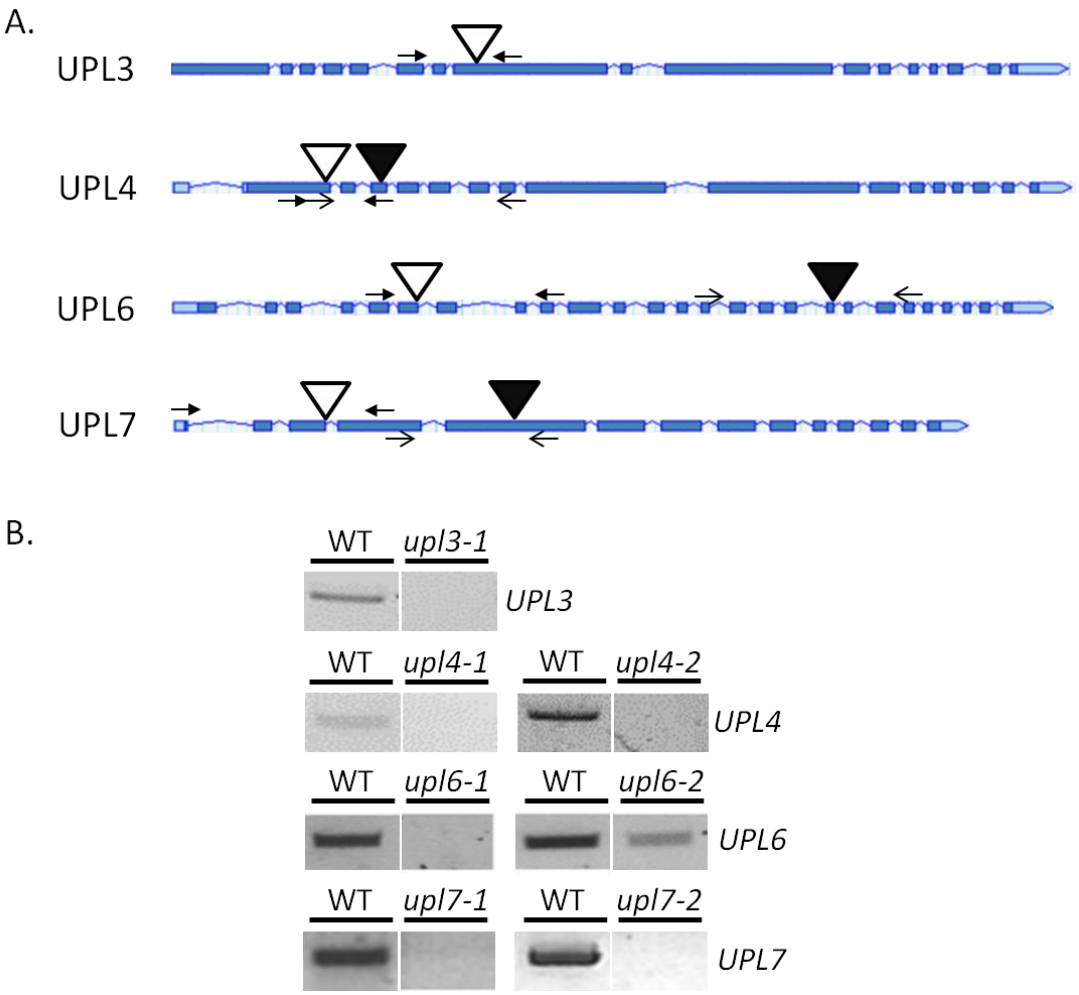

Figure 4.2: All T-DNA insertion alleles have the target gene knocked-out except *upl6-2*.

A. Location of T-DNA insertions in UPL genes. White and black triangles indicate location of T-DNA insertions in allele 1 and 2 of each gene, respectively. Closed and open arrowheads indicate location of primer for alleles 1 and 2 of each gene, respectively. Dark blue rectangles: exons. Lines: introns. Light blue rectangles: UTR. Image modified from Arabidopsis.org. **B.** Total RNA was extracted from indicted wild type and mutant plants and reverse transcriptase-PCR performed with indicated *UPL* gene specific primers.

Figure 4.3: Morphology of T-DNA insertion mutants

Morphology of adult plants at three weeks old. Mutation of UPL3 led to relatively normal growth but misshaped trichomes (not visible in photo) (Downes et al., 2003). The *upl3* upl4 double mutant has much reduced growth relative to the single mutant parents. Scale bar is 1 cm.

4.2.3. UPL3 is a ubiquitin ligase required to maintain total cellular ubiquitination levels

To investigate if the UPLs have global cellular ubiquitin ligase activity, TUBE pull-downs were carried out on seedlings that had been treated with only 0.5 mM SA, a combination of 0.5 mM SA and 100 µM MG132, or water (mock) for 6 hours. In SA-treated wild type global cellular levels of polyubiquitinated proteins and polyubiquitination of the proteasome subunit RPN10 increased slightly, while larger increases were observed in the presence of SA and MG132. A wild type ubiquitination pattern was seen in *upl4-1*, while reduced RPN10 polyubiquitination was occasionally seen in *upl4-2*, but this could not be repeated consistently (Figures 4.4). The most striking differences were observed between

the wild type and *upl3* single or *upl3 upl4* double mutants, which showed reduced levels of cellular polyubiquitination and RPN10 polyubiquitination (Figures 4.4 & 4.5). These data indicate that *upl3* may be a specific ubiquitin ligase of RPN10 and also plays an important role in global cellular ubiquitination.

The total cellular ubiquitination pattern in *upl6* and *upl7* mutants was similar to wild type, however there was a mild reduction in the amount of unmodified RPN10 pulled-down, if not in polyubiquitination, suggesting UPL6 and UPL7 may play a small role in the regulation of RPN10 levels (Figure 4.6 & 4.7).

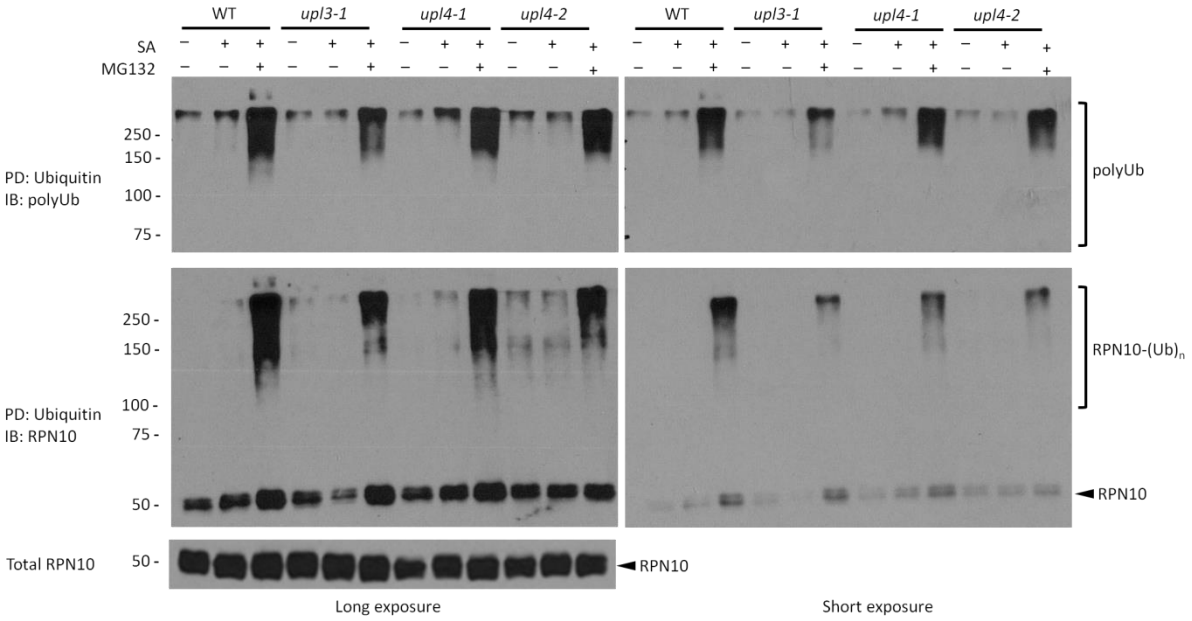

Figure 4.4: UPL3 plays a role in global ubiquitination and the specific ubiquitination of proteasome subunit RPN10.

Approximately 75 14-day old seedlings grown on MS-agar plates were immersed in solutions of DMSO (--), 0.5 mM SA and DMSO (+-) or 0.5 mM SA and 100 μM MG132 dissolved in DMSO (++) for 6 hours before protein extraction in the presence of GST-tagged tandem ubiquitin binding entities (GST-TUBE) to capture ubiquitinated proteins. GST-TUBE was purified on glutathione beads. Eluates and inputs (i.e. Total RPN10) were analysed via SDS-PAGE and immunoblotted (IB) with α-polyubiquitin and α-RPN10 antibodies. Long (left panels) and short (right panels) exposures of blots are shown.

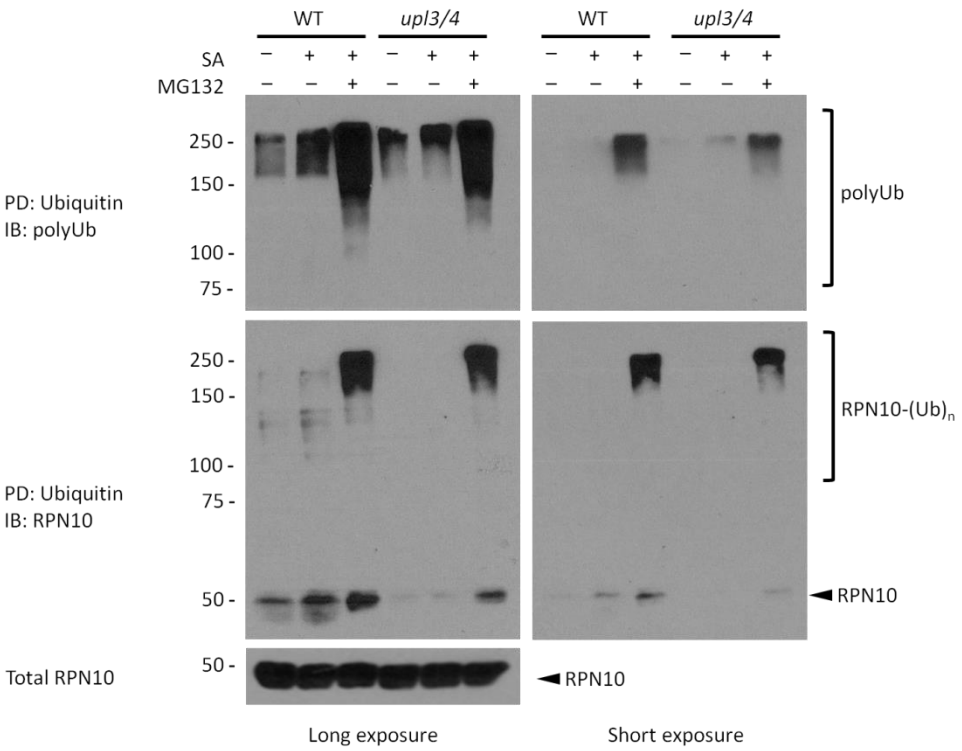

Figure 4.5: The double knockout *upl3 upl4* has reduced global and RPN10-specific polyubiquitination.

Approximately 75 14-day old seedlings grown on MS-agar plates were immersed in solutions of DMSO (--), 0.5 mM SA and DMSO (+-) or 0.5 mM SA and 100 µM MG132 dissolved in DMSO (++) for 6 hours before protein extraction in the presence of GST-tagged tandem ubiquitin binding entities (GST-TUBE) to capture ubiquitinated proteins. GST-TUBE was purified on glutathione beads. Eluate was analysed via SDS-PAGE and probed with α-poly-ubiquitin and α-RPN10 antibodies. Long (left panels) and short (right panels) exposures of blots are shown.

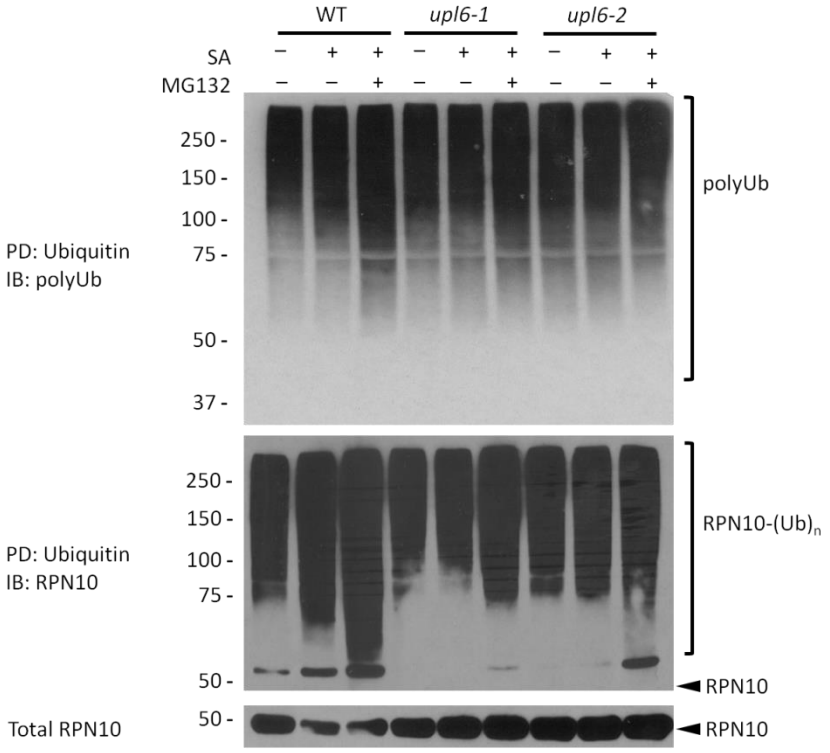

Figure 4.6: UPL6 plays a minor role in global cellular and RPN10-specific polyubiquitination.

Approximately 75 14-day old seedlings grown on MS-agar plates were immersed in solutions of DMSO (--), 0.5 mM SA and DMSO (+-) or 0.5 mM SA and 100 μM MG132 stored in DMSO (++) for 6 hours before protein extraction in the presence of His-tagged tandem ubiquitin binding entities (His-TUBE) to capture ubiquitinated proteins. His-TUBE was purified on His-Pure cobalt beads. Eluates and inputs (i.e. Total RPN10) were analysed via SDS-PAGE and immunoblotted (IB) with α-poly-ubiquitin and α-RPN10 antibodies.

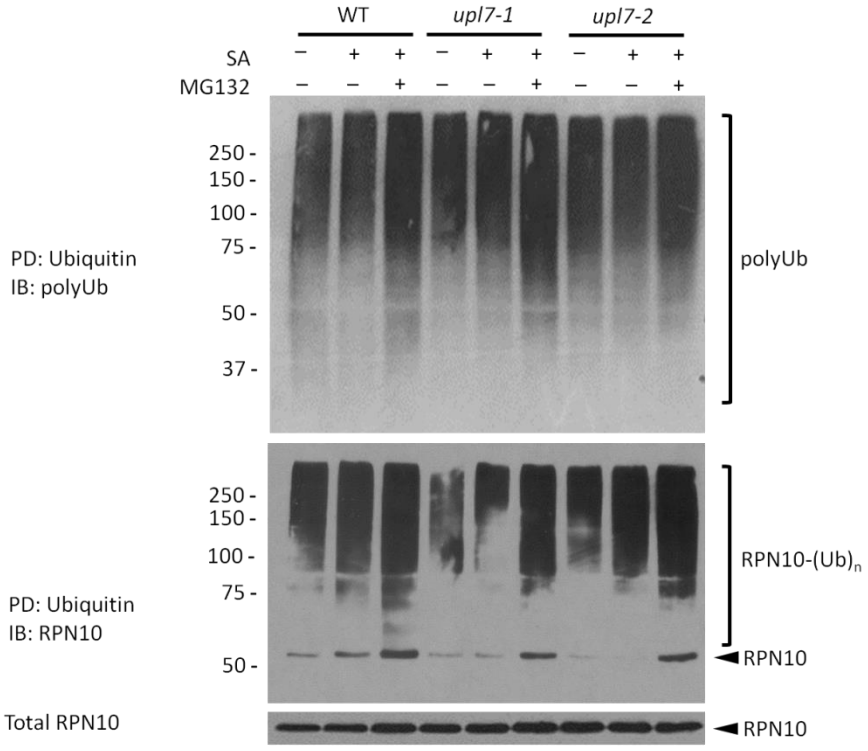

Figure 4.7: UPL7 plays a minor role in global cellular and RPN10-specific polyubiquitination.

Approximately 75 14-day old seedlings grown on MS-agar plates were immersed in solutions of DMSO (--), 0.5 mM SA and DMSO (+-) or 0.5 mM SA and 100 µM MG132 dissolved in DMSO (++) for 6 hours before protein extraction in the presence of His-tagged tandem ubiquitin binding entities (His-TUBE) to capture ubiquitinated proteins. His-TUBE was purified on His-Pure cobalt beads. Eluates and inputs (i.e. Total RPN10) were analysed via SDS-PAGE and immunoblotted (IB) with α-poly-ubiquitin and α-RPN10 antibodies.

4.2.4. Salicylic acid does not induce immune gene expression in *upl3-1* mutants

To test if the ubiquitination patterns seen in *upl* mutants correlates with changes in the transcriptional response to SA, 3-week old plants were sprayed with 0.5 mM SA or water (mock) and leaf samples were collected after 24 hours. After application of SA, expression of all marker genes increased in wild type plants, while in *npr1-1* mutants gene expression did not markedly increase (Figure 4.8). Similar to *npr1-1*, knockout of *UPL3* completely abolished SA-induced expression of *PR* genes. Additionally, expression of most WRKY genes was also reduced in *upl3* mutants. By contrast, the two *upl4* mutant alleles responded with gene expression levels similar to or higher than wild type. Likewise, mutation of *UPL6* or *UPL7* did not result in insensitivity to SA as all mutant alleles showed SA-induced gene expression similar to or higher than wild type (Figure 4.8). These data indicate that of all the *upl* mutants tested, reduced substrate polyubiquitination in *upl3-1* has the most striking effect on SA-responsive immune gene expression.

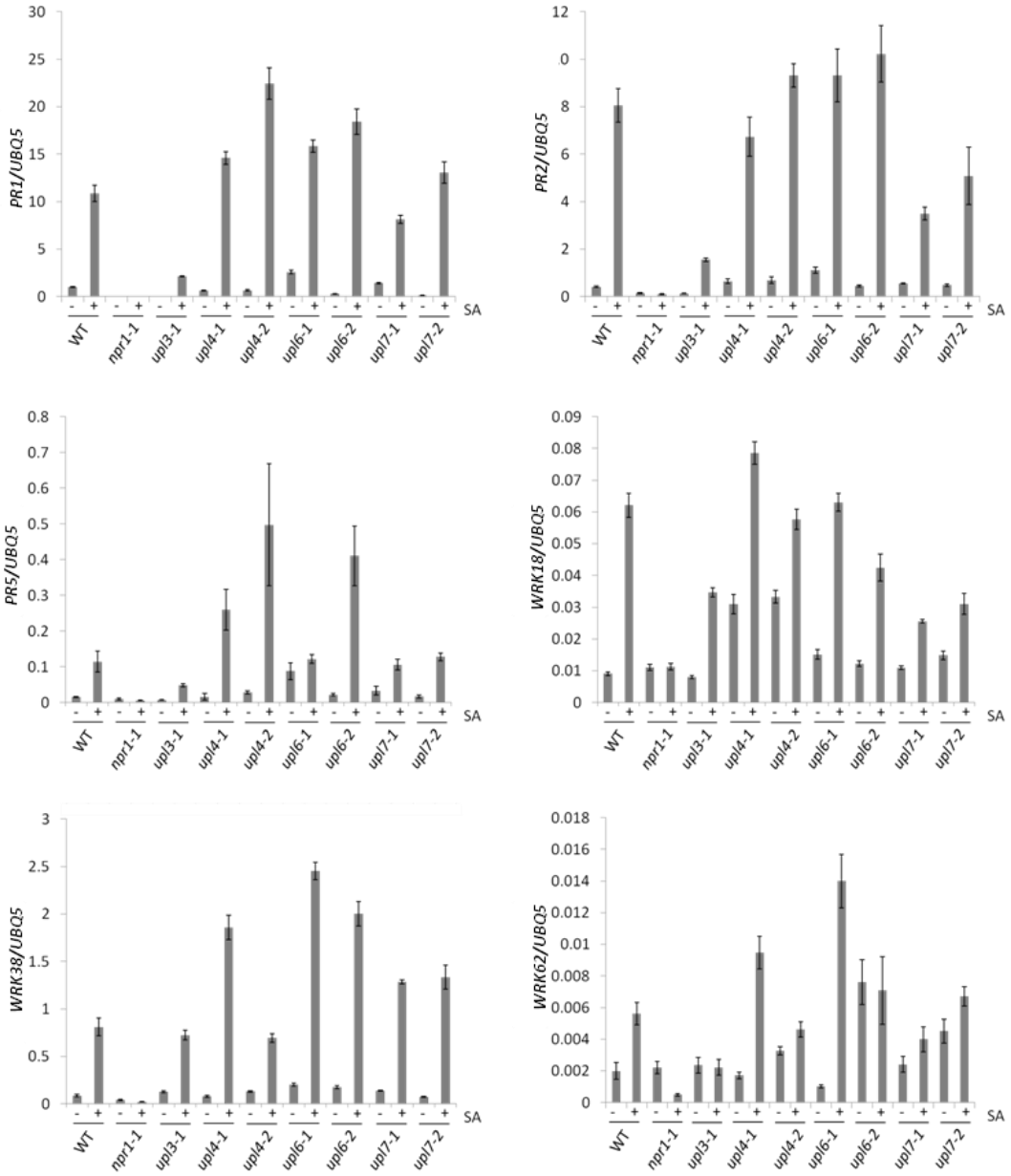

Figure 4.8: SA-induced immune gene expression is impaired in *upl3-1* mutants.

Three-week old plants were sprayed with 0.5 mM SA (+) or water (-) and incubated for 24 h. Two leaves were collected from each plant per treatment and pooled before RNA extraction and qPCR. Expression was normalized against constitutively expressed ubiquitin 5 (UBQ5). n = 3. Error bars indicate standard error from 3 technical replicates.

4.2.5. *UPL* mutants display wild type responses to proteasome inhibition

As UPL proteins are putative ubiquitin ligases, inhibition of the proteasome may reveal proteasome independent gene regulatory functions of the UPLs. Seedlings were immersed in solutions containing only 0.5 mM SA, a combination of SA and 100 µM MG132, or water (mock) for 6 hours. Upon SA treatment *upl3-1* mutants again failed to induce *PR-1* gene expression and also exhibited reduced expression of *WRKY38* (Figure 4.9). Contrary to the previous assay, however, expression of *WRKY18* and *WRKY62* was normal, suggesting that plant age or timing of harvest post application of SA introduced some variation between assays. Nonetheless, this allowed us to study the response of *upl3-1* to proteasome inhibition. Like in the wild type, treatment with MG132 resulted in strong suppression of SA-induced gene expression in *upl3-1* plants (Figure 4.9), indicating that SA-responsive genes still required the proteasome for full expression in this mutant background.

Because UPL4 is closely related to UPL3, we also tested the response of *upl4* mutants to MG132. In *upl4* mutants SA-induced *WRKY* expression was largely equivalent to wild type, except that *PR1* levels were higher. The presence of MG132 also consistently reduced transcription of marker genes in *upl4* mutants to a similar amount as the wild type (Figure 4.9).

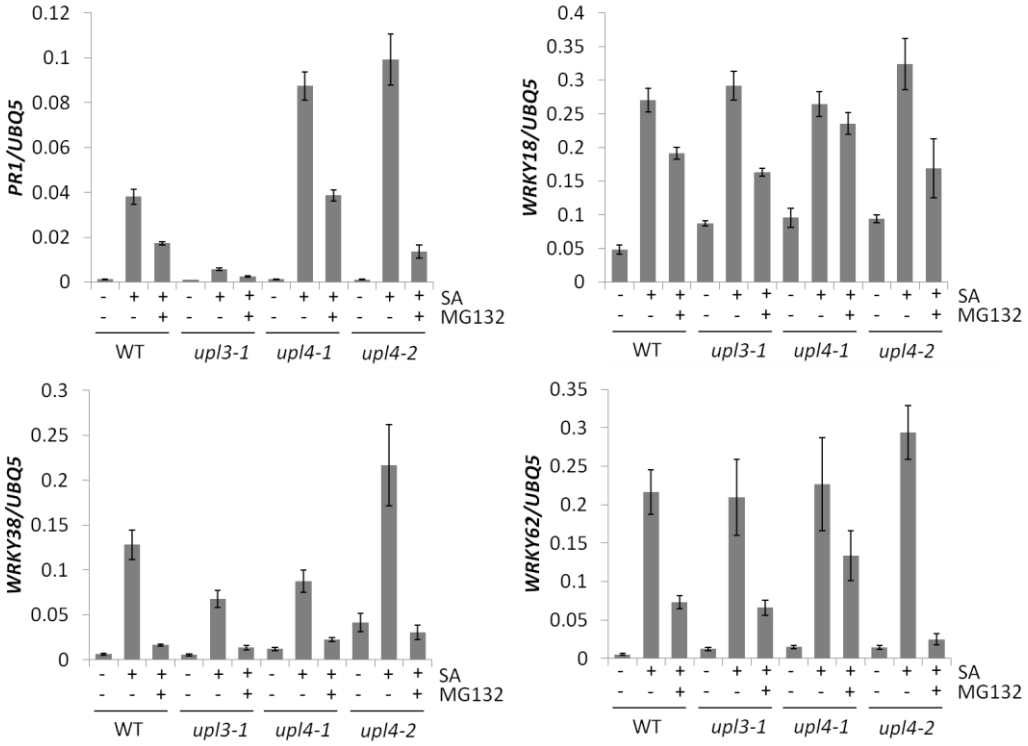

Figure 4.9: SA-responsive gene expression in *upl3* and *upl4* mutants requires proteasome activity.

Approximately 75 14-day old seedlings grown on MS-agar plates were immersed in solutions of DMSO (--), SA and DMSO (+-) or SA and 100 μM MG132 dissolved in DMSO (++) for 6 hours, before RNA extraction and qPCR. Expression was normalized against constitutively expressed ubiquitin 5 (UBQ5). Error bars indicate standard error from 3 technical replicates.

The above experiment was also carried out using *upl6* and *upl7* mutants. Like other *upl* mutants, expression patterns after MG132 treatment were equivalent to that of wild type (Figure 4.10). Hence we conclude that mutation of the different *UPL* genes tested does not influence the dependency of SA-responsive genes on proteasome activity.

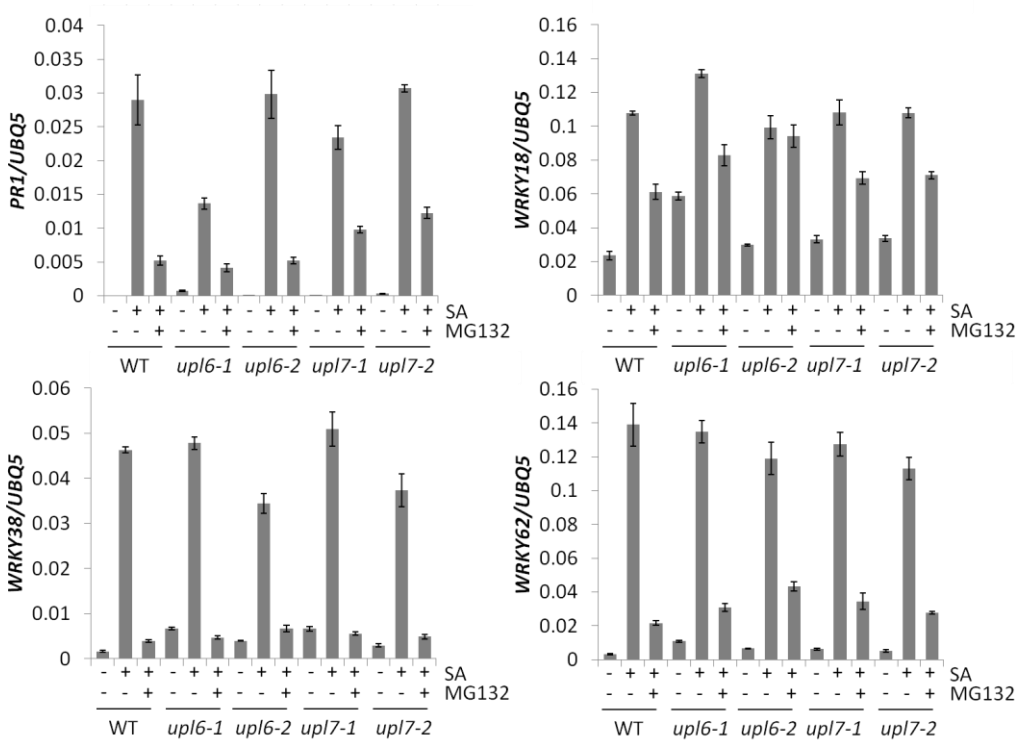

Figure 4.10: SA-responsive gene expression in *upl6* and *upl7* mutants requires proteasome activity.

Approximately 75 14-day old seedlings grown on MS-agar plates were immersed in solutions of DMSO (--), 0.5 mM SA and DMSO (+-) or 0.5 mM SA and 100 µM MG132 dissolved in DMSO (++) for 6 hours, before RNA extraction and qPCR. Expression was normalized against constitutively expressed ubiquitin 5 (UBQ5). Error bars indicate standard error from 3 technical replicates.

4.2.6. The *upl3 upl4* double mutant has a similar transcriptional profile to *upl3-1*

As UPL3 and UPL4 are homologs, it is possible that they operate redundantly masking possible effects of the single gene knock-out; a *upl3 upl4* double mutant was generated by crossing *upl3-1* with *upl4-2.* Seedlings were immersed in 0.5 mM SA for 24 hours and SA-responsive gene expression analysed. The *upl3 upl4* double mutant showed a similar transcriptional profile to the *upl3-1* single mutant, in that *PR-1* gene expression was strongly reduced. However, expression of *WRKY18* and *WRKY38* was similar to wild type and that of *WRKY62* was higher (Figure 4.11). As expression levels in the double mutant were comparable to the *upl3-1* single mutant, this indicates that mutation of UPL4 does not further aggravate mutant phenotypes.

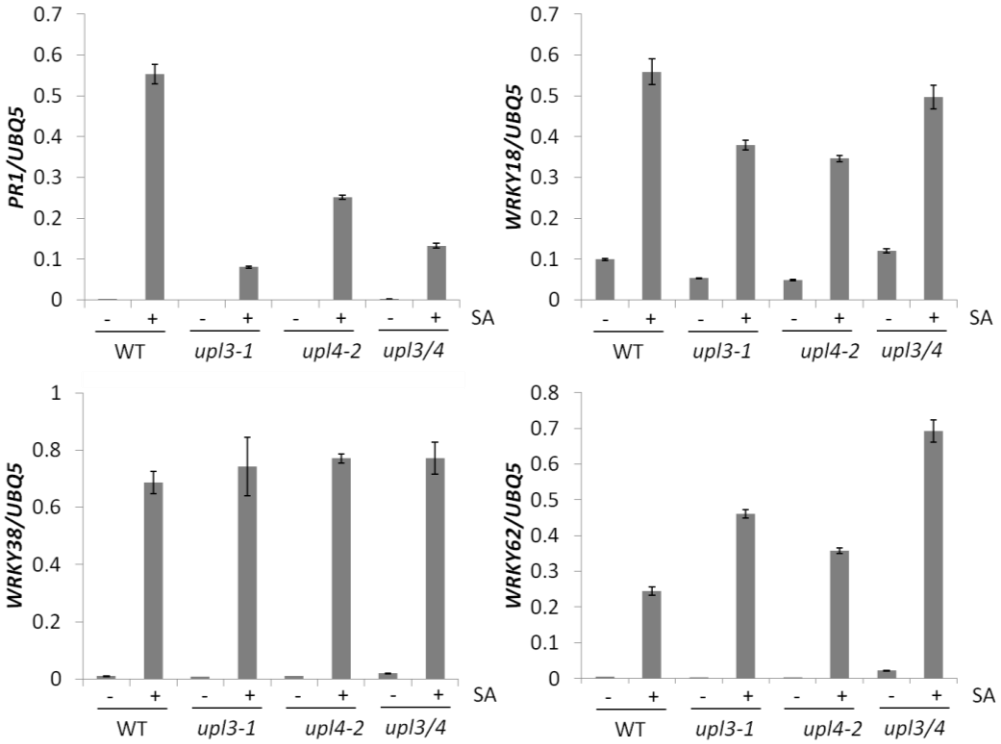

Figure 4.11: Knockout of both UPL3 and UPL4 prevent correct expression of PR genes.

Approximately 75 14-day old seedlings grown on MS-agar plates were immersed in a 0.5 mM SA solution (+) or water (-) for 24 hours, before RNA extraction and qPCR. Expression was normalized against constitutively expressed ubiquitin 5 (UBQ5). Error bars indicate standard error from 3 technical replicates.

4.2.7. Mutation of *UPL3* or *UPL4,* but not *UPL6* and *UPL7* alters disease resistance

As *upl3-1* has a reduced capacity to express *PR* genes in response to SA (Figures 4.8, 4.9 & 4.11) an enhanced disease resistance test was performed to assess if it was biologically relevant. Wild type plants which had been pre-treated with SA before inoculation with the virulent pathogen *Psm* ES4326 had a significantly reduced amount of pathogen growth. Mutation of *UPL3* led to greater pathogen growth than untreated wild type and application of SA could not reduce pathogen growth (Figure 4.12). Plants in which *UPL4* was knocked out had the same level of basal defence as wild type, but were not as resilient to pathogen infection after SA treatment (Figure 4.12). Moreover, mutation of *UPL6* or *UPL7* had no effect on disease resistance with or without the presence of SA (Figure 4.13). These data indicate that of all UPL proteins, UPL3 plays the most important role in SA-dependent plant immune responses.

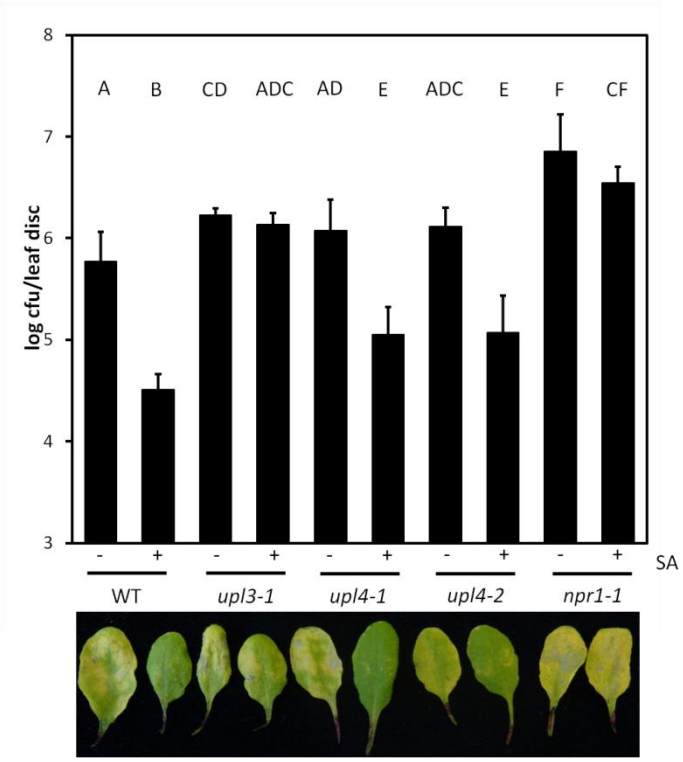

Figure 4.12: The SA-mediated defence response against a virulent pathogen is compromised in *upl3-1.*

Enhanced disease resistance test: Top: Quantification of pathogen levels within infected leaves. Bottom: Disease symptoms of infected leaves. Three-week old wild type, *upl3, upl4* and *npr1-1* plants were sprayed with water (-) or 0.5 mM SA (+) and incubated for 24 hours before syringe infiltration with 5 x 10^6 colony forming units/ml *Psm* ES4326 into the abaxial surface of a leaf. Leaf discs were collected 4-days post inoculation, bacteria extracted and serial diluted before colony counting on selective agar plates. Error bars indicate 95 % confidence limits (n = 8). Letters indicate significantly different samples (Tukey-Kramer ANOVA test, $\alpha = 0.05$, n = 8).

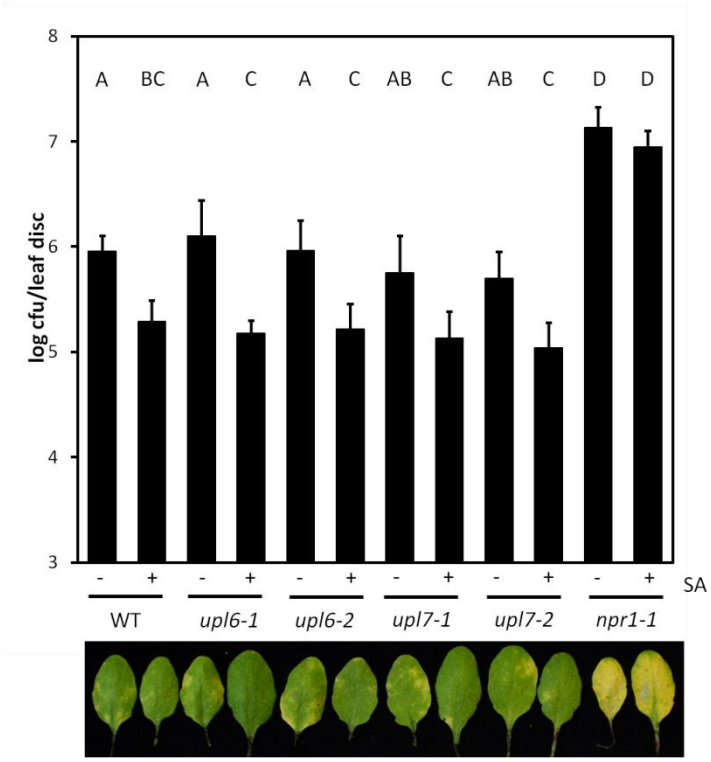

Figure 4.13: The *upl6* and *upl7* mutants have wild type disease resistance responses.

Enhanced disease resistance test: Top: Quantification of pathogen levels within infected leaves. Bottom: Disease symptoms of infected leaves. Three-week old wild type, *upl6, upl7* and *npr1-1* plants were sprayed with water (-) or 0.5 mM SA (+) and incubated for 24 hours before syringe infiltration with 5 x 10^6 colony forming units/ml *Psm* ES4326 into the abaxial surface of a leaf. Leaf discs were collected 4-days post inoculation, bacteria extracted and serial diluted before colony counting on selective agar plates. Error bars indicate 95 % confidence limits (n= 8). Letters indicate significantly different samples (Tukey-Kramer ANOVA test, $\alpha = 0.05$, n = 8).

4.2.8. Basal immune responses are compromised in *upl3* and the *upl3 upl4* double mutant

Both *upl3* and *upl4* plants acted different from wild type when primed with SA and then challenged with a high inoculum dose (5 x 10^6 colony forming units/ml) of virulent *Psm* ES4326 pathogen (Figure 4.11). Instead of this induced immunity, we also tested basal immune defences by using a 10-fold lower inoculum concentration (5 x 10^5 colony forming units/ml) of virulent *Psm* ES4326. While wild type plants exhibit no symptoms and complete resistance against *Psm* ES4326 at this low inoculum, mutants such as *npr1-1* are clearly susceptible, showing leaf yellowing and extensive pathogen growth (Figure 4.14). While *upl4* mutants showed similar basal immunity as wild type, *upl3-1* mutants were more susceptible to *Psm* ES4326. Surprisingly, double mutant *upl3 upl4* plants allowed even higher pathogen growth and associated chlorosis than *npr1-1*, indicating that both NPR1-dependent and NPR1-independent defences were abolished in this background (Figure 4.14). By contrast, *upl6* and *upl7* single mutants as well as *upl6 upl7* double mutants displayed wild type levels of disease growth, indicating the mutations did not affect disease resistance (Figure 4.15).

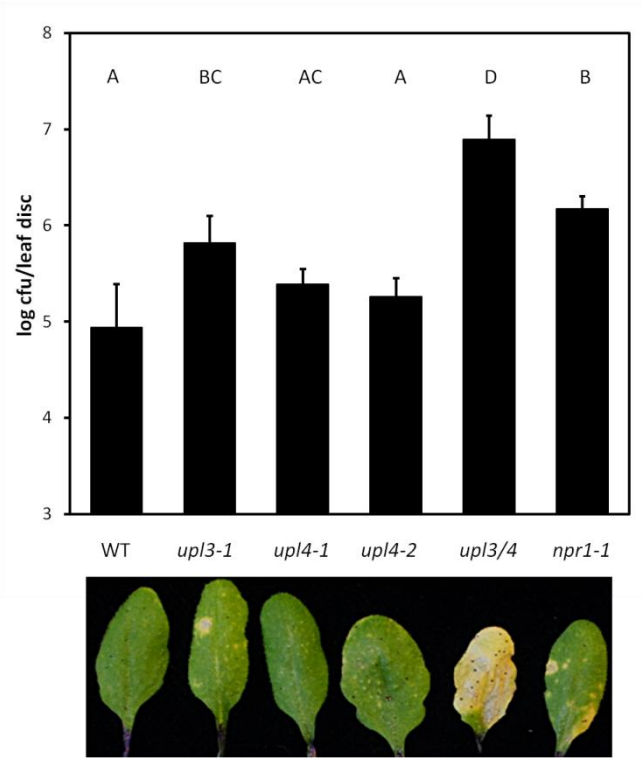

Figure 4.14: Disease susceptibility is increased in *upl3-1* single and *upl3 upl4* double mutants

Enhanced disease susceptibility test: Top: Quantification of pathogen levels within infected leaves. Bottom: Disease symptoms of infected leaves. Three-week old adult plants were inoculated via syringe infiltration with 5 x 10^5 colony forming units/ml *Psm* ES4326 into the abaxial surface of a leaf. Leaf discs were collected 4-days post inoculation, bacteria extracted and serial diluted before colony counting on selective agar plates. Error bars indicate 95 % confidence limits (n = 8). Letters indicate significantly different samples (Tukey-Kramer ANOVA test, $\alpha = 0.05$, n = 8).

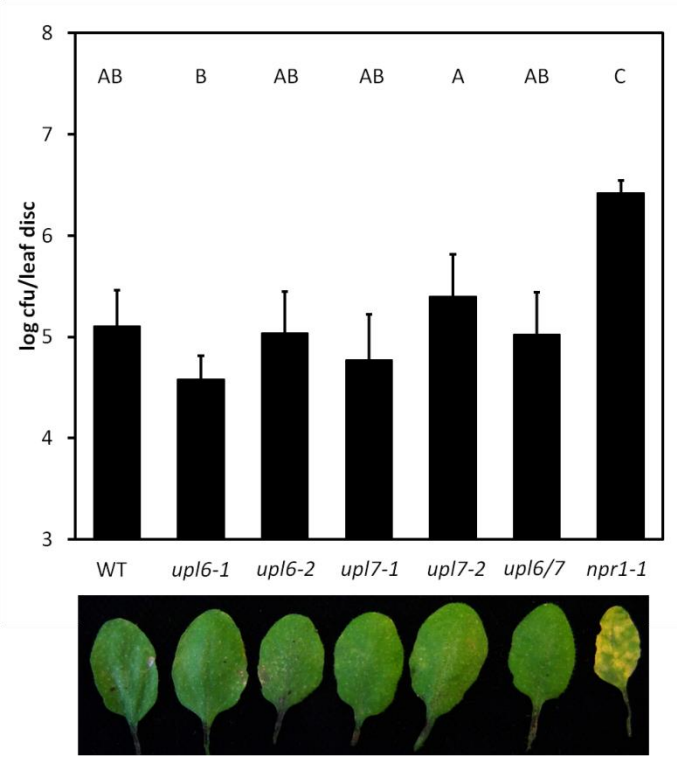

Figure 4.15: The *upl6* and *upl7* mutants do not have significantly altered disease resistance.

Enhanced disease susceptibility test: Top: Quantification of pathogen levels within infected leaves. Bottom: Disease symptoms of infected leaves. Three-week old adult plants were inoculated via syringe infiltration with 5 x 10^5 colony forming units/ml *Psm* ES4326 into the abaxial surface of a leaf. Leaf discs were collected 4-days post inoculation, bacteria extracted and serial diluted before colony counting on selective agar plates. Error bars indicate 95 % confidence limits (n = 8). Letters indicate significantly different samples (Tukey-Kramer ANOVA test, $\alpha = 0.05$, n = 8).

4.2.9. UPL3 is required for correct regulation of the SA-dependent transcriptome

Knockout of *UPL3* produced plants that were compromised in both basal and SA-induced disease resistance (Figures 4.11 & 4.13). Accordingly SA-induced *PR* gene expression was clearly abolished in *upl3-1* plants but the effect on other genes such as the *WRKY* markers was less clear (Figures 4.7 & 4.8). To compose a clearer picture of how mutation of *UPL3* leads to partial insensitivity to SA and disease susceptibility, RNA-sequencing (RNA-seq) was performed on adult WT and *upl3-1* that had been sprayed with 0.5 mM SA or water (mock) and incubated for 24 hours. We included 3 biological replicates for which total mRNA was purified and sent to GATC Biotech (Konstanz, Germany) for RNA-seq and bioinformatics analyses.

Differences in gene expression between the wild type and *upl3-1* genotypes were immediately apparent, as *upl3* had a much smaller amount of genes with significantly altered expression after SA treatment (Figure 4.16). Scatter plots illustrated that SA-treated wild type plants had a greater number of genes that responded to SA and a larger spread in the fold change of gene expression compared to *upl3-1* (Figure 4.17). Indeed, large scale changes in gene expression were initiated after induction with SA in wild type plants with 6419 genes significantly altered in expression; this is 23.3 % of the 27,416 protein coding genes in *Arabidopsis* (Swarbreck et al., 2008). Knockout of *UPL3* resulted in an inability to muster such a large scale response to SA with a significant change detected in only 2,443 genes or 8.9 % of the genome (Figure 4.16).

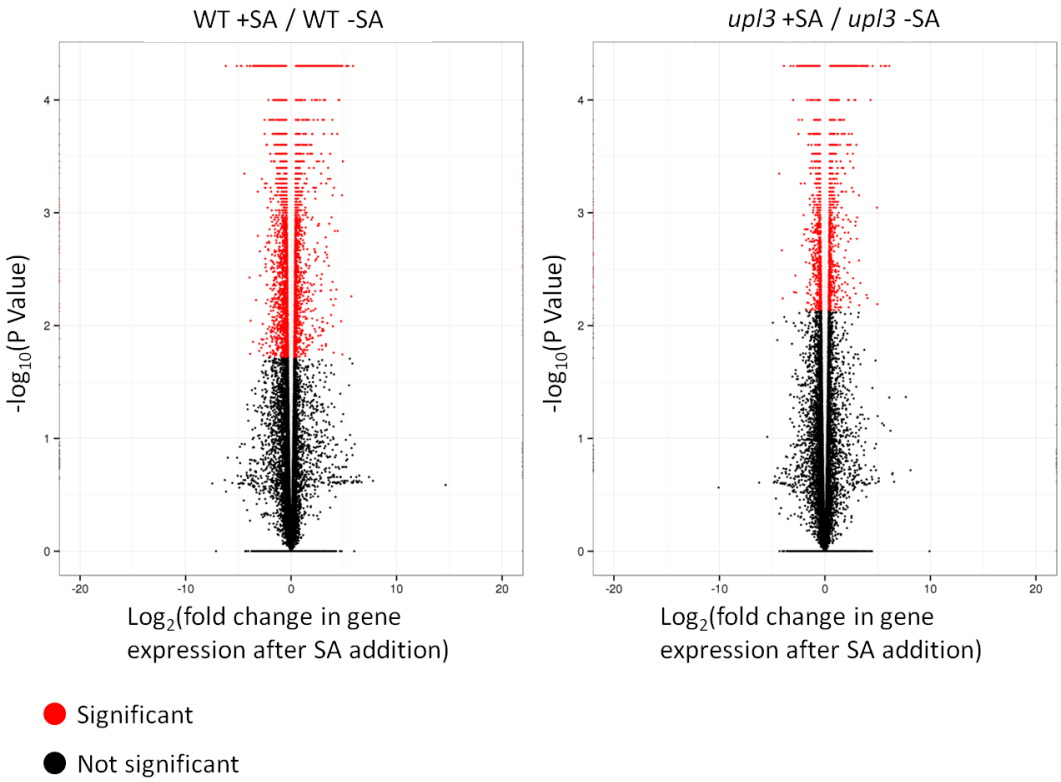

Figure 4.16: SA treatment activates fewer genes with significant fold change in *upl3-1.*

Volcano plots of fold change in gene expression after SA induction in wild type (left) and *upl3-1* (right). Dots indicate a genes fold change in expression in response to SA induction versus P-value. Significant if q-value < 0.05.

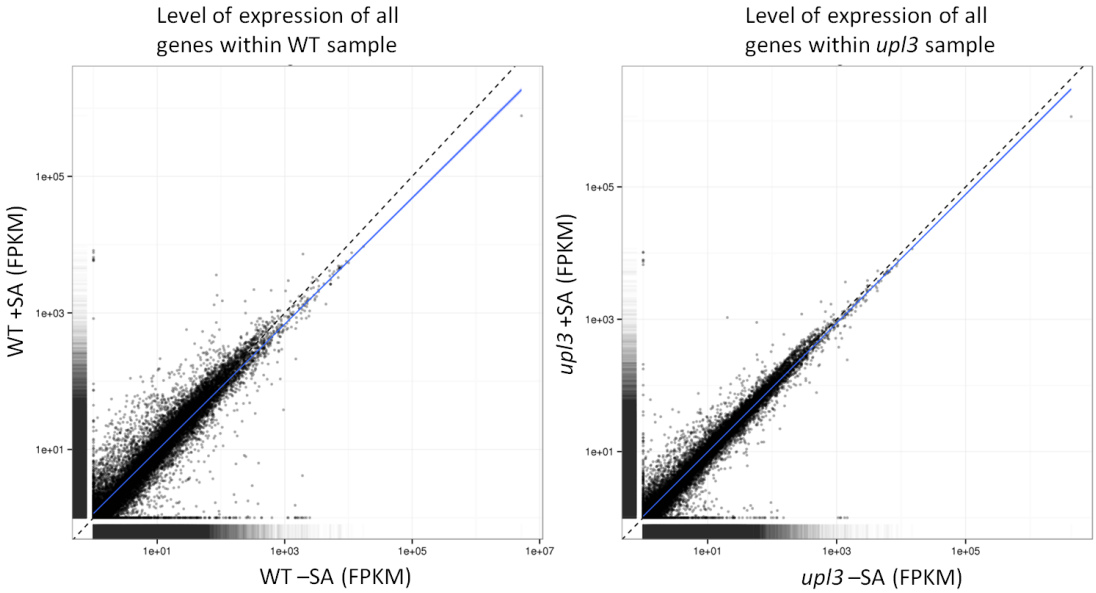

Figure 4.17: SA treatment induces larger numbers of genes and changes in fold expression in wild type than *upl3-1*.

Scatter-plots of gene expression with (+) and without (-) SA induction in wild type (left) and *upl3-1* (right). Dots indicate read count as measured by fragments per kilobase per million reads mapped (FPKM). Dotted line: X axis = Y axis. Solid line: Trend line of samples (X vs Y).

As the amount of genes which had significant changes in gene expression after SA induction was of considerable size, we decided to make the data set more stringent and focus on those genes with a large change gene in expression level; therefore we applied a cut-off of a minimum of two-fold change in expression. Wild type had 1918 genes with a two-fold change in expression, whereas *upl3* had only 658 genes. Changes in two-fold or greater gene expression were roughly evenly split between those genes that were upregulated and those that were down-regulated by SA in wild type, but *upl3* had almost half as many genes down-regulated compared to upregulated (Figure 4.18). Although there were a greater number of genes expressed only in wild type, most genes expressed in *upl3-1* genes overlapped with those in wild type, indicating this mutant was able to activate a small sub-set of the correct genes needed for the SA response. In addition to those genes that *upl3-1* did not express, 130 genes were expressed to a significant level exclusively in *upl3-1* (Figure 4.18), indicating that as well as lacking the ability to adjust the transcription of genes required for the SA response, there was a considerable amount of off-target expression changes.

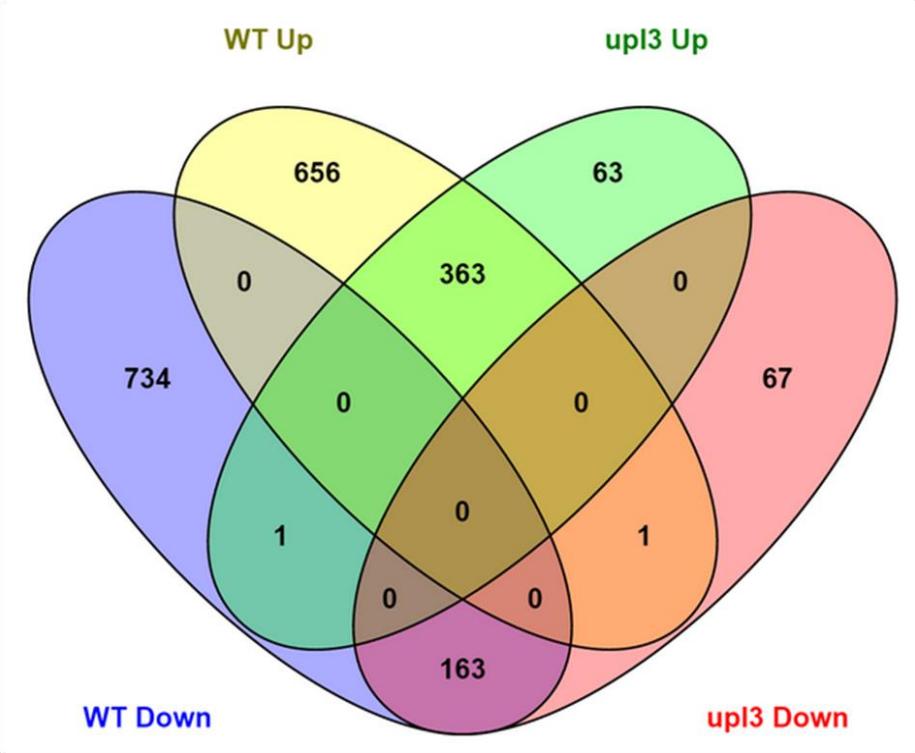

Figure 4.18: Distribution of significant differential gene expression changes in response to SA.

A Four-way Venn diagram of genes with a greater than two-fold change in gene expression, after SA induction in wild type (WT) and *upl3-1.* Up: genes up-regulated after SA induction. Down: genes down-regulated after SA induction. Diagram drawn using Venny 2.0 (Oliveros, 2007-2015).

Using the top 50 genes up- and down-regulated by SA in WT, we generated a heat map to observe differential expression between wild type and *upl3-1*. This clearly demonstrated that *upl3-1* was compromised in the SA response, as generally it was not able to initiate the level of fold change as seen in the wild type (Figure 4.19).

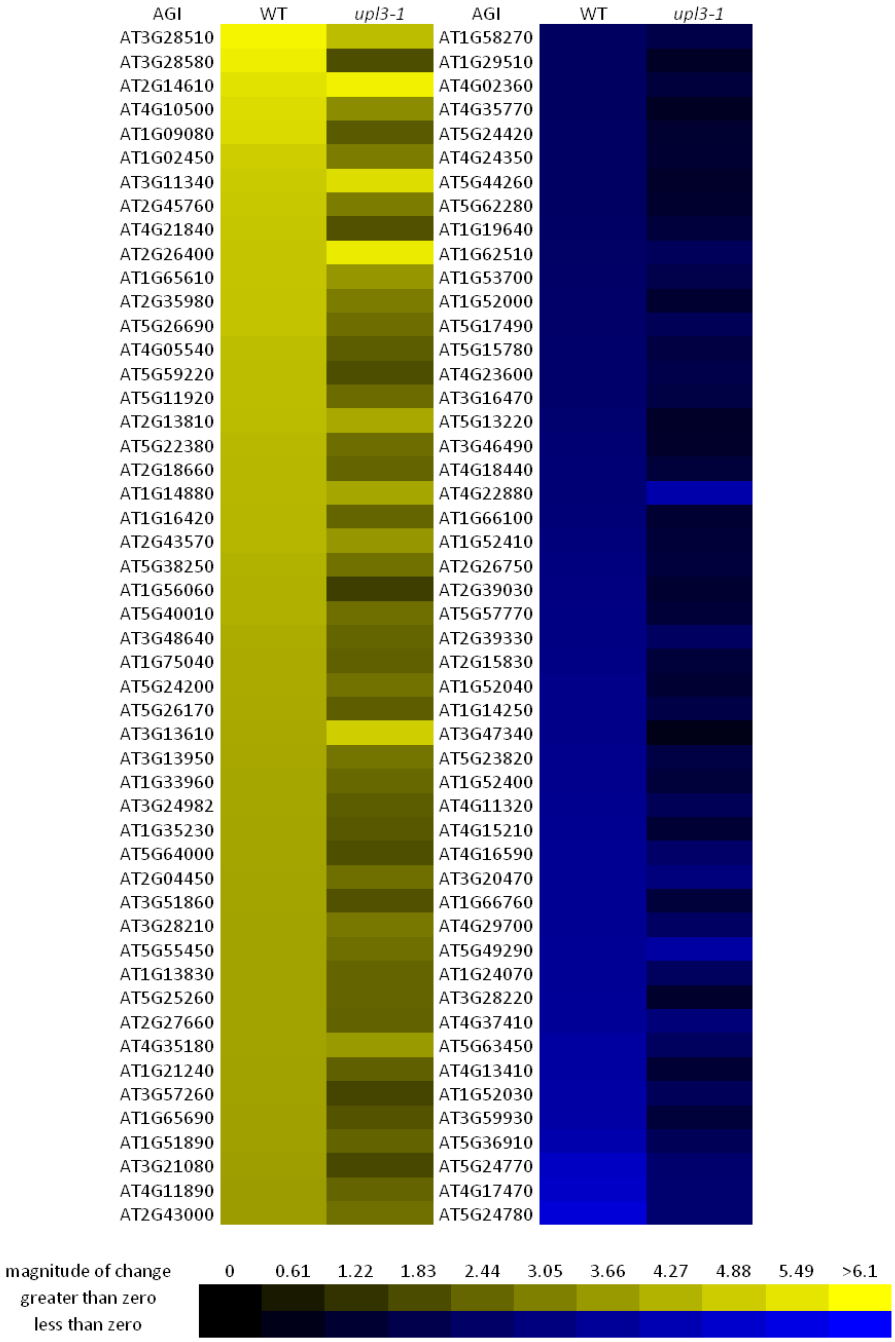

Figure 4.19: Mutation of UPL3 prevents correct level of gene expression following induction with SA.

Heat map of the log2 fold change of the top 50 up-regulated and down-regulated (yellow and blue, respectively) genes in wild type compared to *upl3-1.* Heat map was built using Heatmapper at BAR.utoronto.ca.

4.2.10. UPL3-dependent gene expression also requires NPR1

As NPR1 is the regulator for over 98 % of SA-dependent gene induction (Wang et al., 2006) we asked if any SA-induced genes were independent of NPR1 but required UPL3. To generate the list of SA-mediated and UPL3- dependent genes, we compared those genes with two-fold or greater change in expression after SA induction in wild type, against a list of genes with a 1.5-fold difference in expression between wild type and *upl3* after SA induction. By comparing the UPL3-dependent genes discovered in our RNA-seq data to publically available microarray data, which used the SA analogue BTH to induce expression in wild type and *npr1-1* (Wang et al., 2006), we found that the vast majority of genes which were found to be UPL3-dependent and BTH-dependent also required NPR1 (Figure 4.20) therefore UPL3 probably acts in an NPR1-dependent pathway.

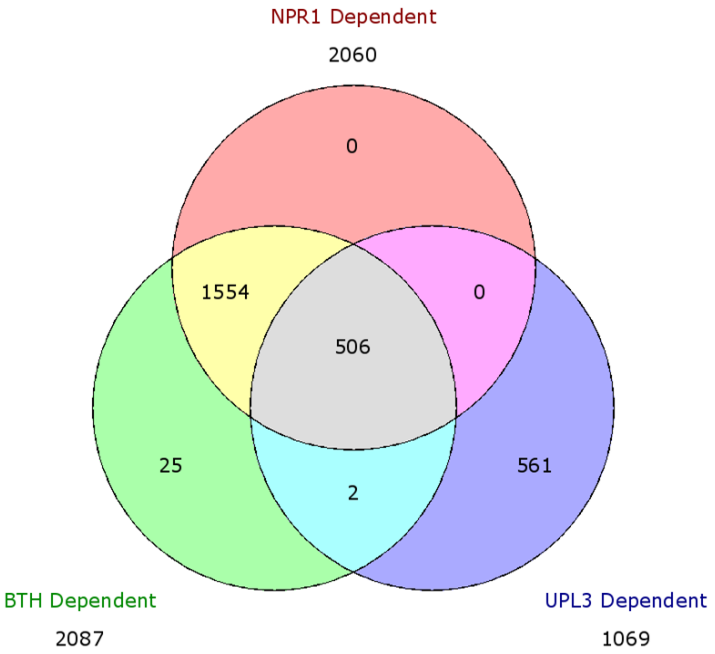

Figure 4.20: SA induced UPL3-dependent gene expression also requires NPR1.

UPL3-dependent genes were compared to genes found to have significant changes in expression levels after application of SA analog BTH from public microarray data (Wang et al., 2006). ATG numbers from experiments were compared using a Venn diagram builder at http://www.bioinformatics.lu/venn.php.

4.2.11. Promoter analysis reveals UPL3 may target WRKY genes

SA-dependent gene expression mediated by NPR1 is also positively and negatively regulated by the WRKY group of transcription factors (Wang et al., 2006) and UPL3 had been shown to interact with and degrade WRKY53 (Patra et al., 2013). Therefore, we decided to investigate if WRKYs are a general target of UPL3. To accomplish this we used the UPL3-dependent gene list described above and applied a p-value of 0.05 and a q-value cut-off of <0.01 to filter out false positives. A cis-promoter analysis was performed on the top 200 genes which were down-regulated in *upl3* compared to wild type to discover the most common pentamers within the promoter regions. This analysis revealed that the most

common motif was the core of the W-box WRKY binding sequence (T)(T)TGAC(C/T) (Figure 4.21).

To confirm this analysis and compare the prevalence of the W-box between SA-regulated genes that were significantly down- and up-regulated in *upl3-1* versus wild type, we then ran the first 1000 bp upstream of the transcription start site of the top 55 genes with the greatest up- and down-regulated expression changes through the POBO promoter analysis tool (Kankainen and Holm, 2004). In genes down-regulated in *upl3-1* versus wild type the WRKY motif was over-represented, while in the genes up-regulated in the *upl3-1* versus wild type the WRKY motif was under-represented (Figure 4.22).

The cis-promoter analysis was repeated for those genes up-regulated in *upl3* versus wild type, revealing that the CACATG variant of the E-box (CANNTG) was a highly common motif along with the closely related G-box (CACGTG) (Figure 4.23). POBO analysis confirmed that the CACATG motif was over-represented in the *upl3* up-regulated genes (Figure 4.24). Taken together, these data show that UPL3 is required to provide full activation of WRKY genes in response to SA induction and also plays an important role in SA-mediated suppression of genes via the CACATG variant of the E-box motif.

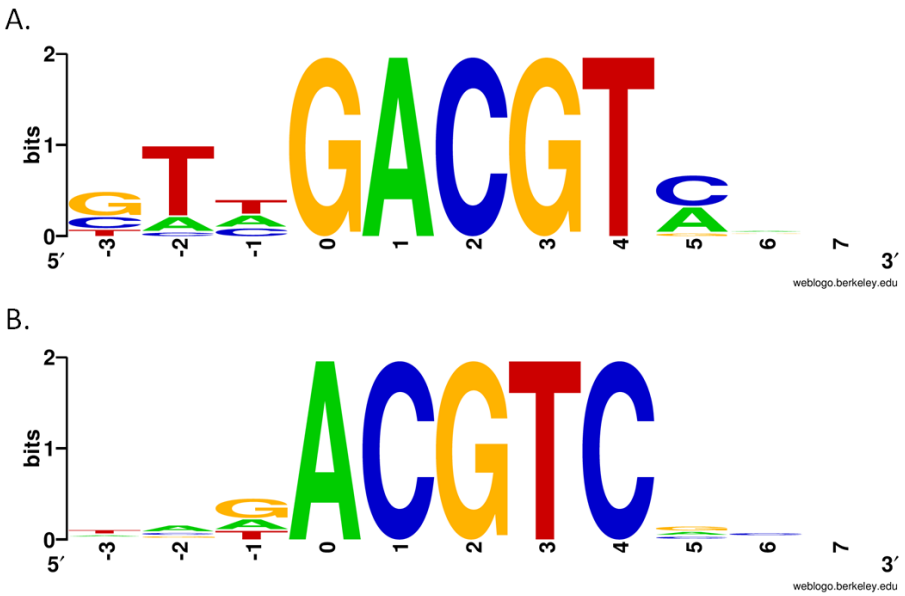

Figure 4.21: The W-box is the most prevalent TF binding motif domain in SA-induced genes that are down-regulated in *upl3* versus wild type.

The first (A) and second (B) most common representations of the W-box found in genes down-regulated in response to SA in *upl3* versus wild type. Cis-promoter analysis was performed on the top 200 genes down-regulated in *upl3-1* compared to wild type (p = 0.05, q = 0.01). All possible 8 bp (octamer) combinations were first calculated to obtain relative appearance ratio, comparing the promoter regions of selected gene set against all genes in *Arabidopsis*. The most enriched pentamers were subsequently calculated from the octamers. Weblogo analysis was performed using pentamers plus the adjacent sequences in the selected gene set. Cis-promoter and weblogo analysis was performed in collaboration with the laboratory of Prof. Yasuomi Tada at Nagoya University.

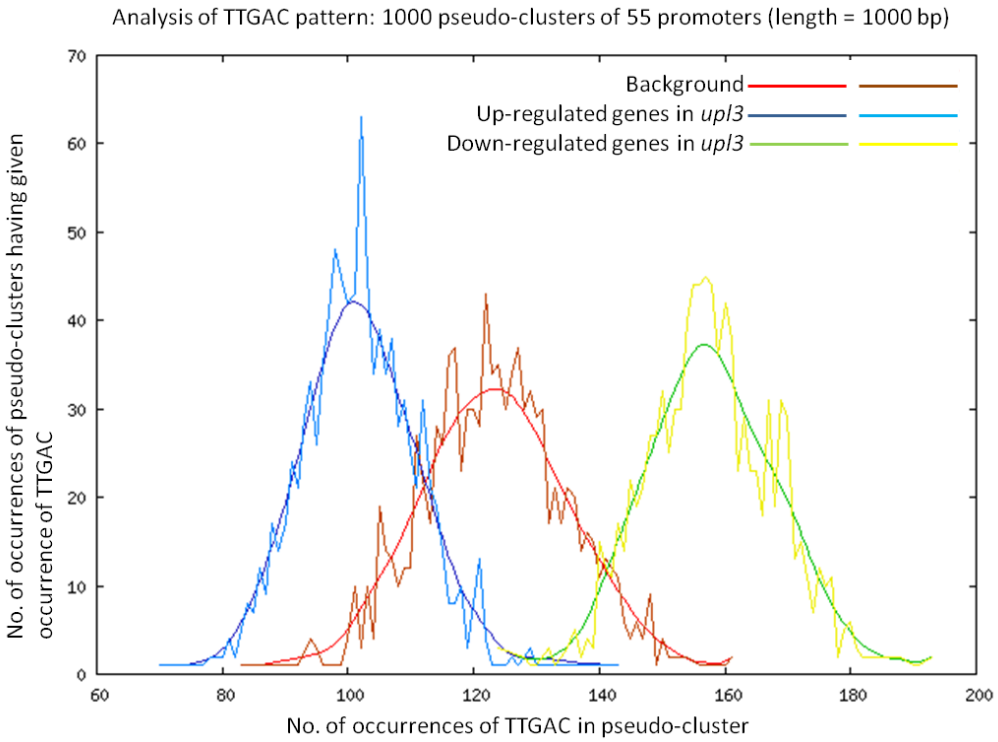

Figure 4.22: The WRKY binding site is over-represented in SA-induced genes that are down-regulated in *upl3* versus wild type.

The 1000 bp upstream of the TAIR10 loci of the top 55 UPL3-dependent genes up- and down regulated in *upl3-1* compared to wild type (q-value < 0.001) were analysed via the promoter analysis tool POBO (Kankainen and Holm, 2004). T-value down-regulated vs background = 67.73. T-value up-regulated vs background = -45.44. T-value between up-regulated vs down-regulated = 125.88.

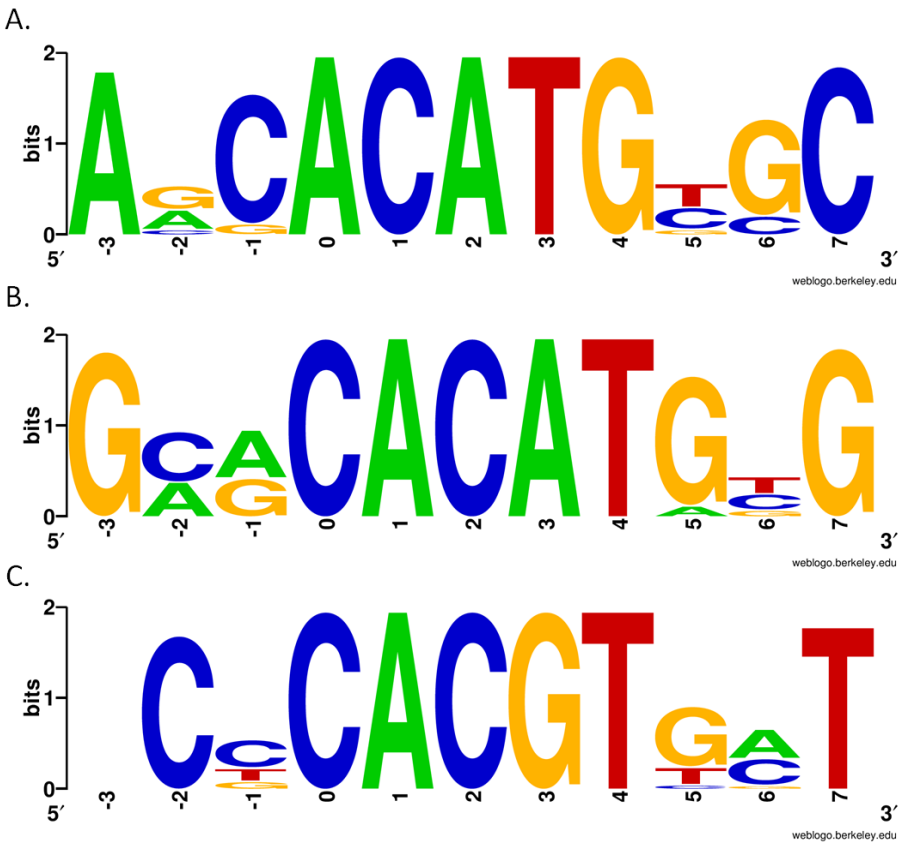

Figure 4.23: The E-box variant CACAGT and the related G-box are prevalent TF binding motifs in SA-repressed genes that are up-regulated in *upl3* versus wild type.

The first (A) and second (B) most common representations of the CACAGT motif and the most common representation of the G-box (C) in SA-repressed genes that were up-regulated in *upl3* versus wild type. Cis-promoter analysis was performed on the top 200 genes up-regulated in *upl3-1* compared to wild type (p = 0.05, q = 0.01). All possible 8 bp (octamer) combinations were first calculated to obtain relative appearance ratio, comparing the promoter regions of selected gene set against all genes in *Arabidopsis*. The most enriched pentamers were subsequently calculated from the octamers. Weblogo analysis was performed using pentamers plus the adjacent sequences in the selected gene set. Cis-promoter and weblogo analysis was performed in collaboration with the laboratory of Prof. Yasuomi Tada at Nagoya University.

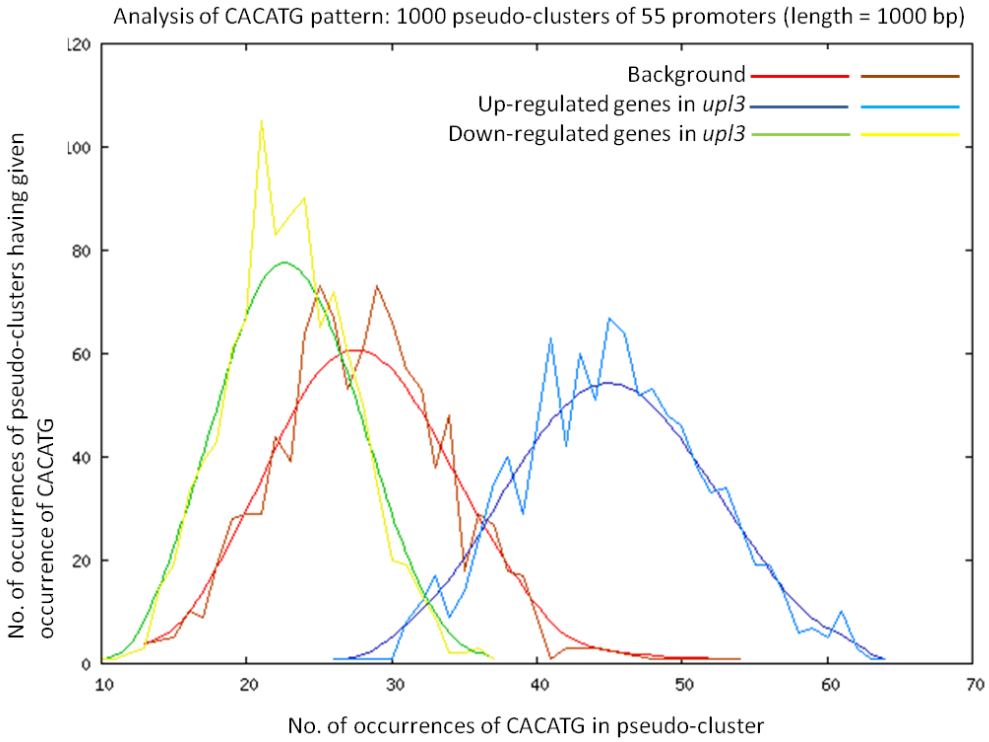

Figure 4.24: The CACATG variant of the E-box (CANNTG) is over-represented in SA-repressed genes that are up-regulated in *upl3* versus wild type.

The 1000 bp upstream of the TAIR10 loci of the top 55 UPL3-dependent genes up- and down regulated in *upl3-1* compared to wild type (q-value < 0.001) were analysed via the promoter analysis tool POBO (Kankainen and Holm, 2004). T-value down-regulated vs background = -21.85. T-value up-regulated vs background = 62.02. T-value between down-regulated vs up-regulated = -89.38.

4.3. Discussion

In this chapter, we used sequence alignment and homology modelling to the yeast E4 ligase, HUL5, to identify *Arabidopsis* UPL proteins as putative ubiquitin ligases. Several UPL proteins were investigated for ubiquitin ligase activity and defects in SA-mediated transcription. Using this approach we identified UPL3 as being required to maintain cellular regulation of ubiquitination and provide SA-dependent disease resistance. RNA-sequencing revealed that *upl3* mutants were unable to induce large-scale transcriptomic changes in response to SA, demonstrating that UPL3 is a crucial and novel ubiquitin ligase within SA-mediated gene transcription and immunity.

The UPL genes were selected because they showed homology to the yeast E4 ligase HUL5, which is a proteasome-bound E4 ligase that acts in opposition to the deubiquitinating enzyme UBP6 (Crosas et al., 2006). Deletion of HUL5 leads to decreased polyubiquitination and stabilisation of proteins, while the opposite effect is seen in *ubp6Δ*. Therefore the balance of ubiquitination is thought to be vital to the regulation of substrate turnover (Crosas et al., 2006). UPL3 is also a putative homolog of the yeast E3/E4 ligase UFD4 (Supplemental 4.2). Like HUL5, UFD4 is localized to the proteasome and contributes to polyubiquitnation and degradation of substrates (Xie and Varshavsky, 2002). Similar to UPL3, UFD4 also contains Arm repeats (Supp) (Ju et al., 2007). UFD4 uses the Arm repeats as binding sites to associate with ubiquitinated substrates and extend their ubiquitin chains (Ju et al., 2007). UPL3 has been associated with trichome development; with the Arm domains of UPL3 binding the bHLH and ACT domains of the transcription factors GL3 and EGL3 and mediating ubiquitination leading to proteasomal degradation of GL3 and EGL3 (Downes et al., 2003, Patra et al., 2013). UFD4 is also required for specific ubiquitination events, including the regulation of RAD25 involved in nuclear excision repair (Bao et al.,

2015). UFD4 was also shown to cooperate with the RING E3 ligase, ubiquitin n-recognin 1 (UBR1), in increasing the processivity of substrate turnover in the ubiquitin-fusion-degradation pathway and proteins involved in the N-end rule pathway, unifying two systems of proteasome-mediated degradation (Hwang et al., 2010). These reports suggest UPL proteins in *Arabidopsis* may have the potential to fulfil the roles of E3 or E4 ligases depending upon the molecular context.

UPL6 and UPL7 do not have a role within SA-mediated plant defences as mutation of these genes and the double mutant has no discernible effect on SA responses: displaying wild type gene expression (Figures 4.8 & 4.10), disease susceptibility (Figure 4.15) and resistance (Figure 4.13), as well as normal ubiquitination patterns (Figures 4.6 & 4.7). Instead, UPL3 is shown here to act as a ubiquitin ligase, as *upl3* single and *upl3 upl4* double mutants had reduced polyubiquitination at the total cellular level and of a specific substrate, RPN10 (Figures 4.4 & 4.5). Reduction of global cellular polyubiquitination is an unusual phenotype as most E3 ligases have very specific targets and therefore have no discernible effect on total polyubiquitination levels. However, E4 ligases are less specific as they act on substrates on which initial rounds of ubiquitination have already been undertaken. Deletion of the yeast E4 ligases, UFD2 and HUL5, did lead to a general reduction of polyubiquitinated substrates (Koegl et al., 1999, Crosas et al., 2006). Like E4 ligases, DUBs also display reduced target specifity compared to E3s and can alter the balance of total polyubiquitination (Swaminathan et al., 1999, Amerik et al., 2000, Crosas et al., 2006). Deletion of HUL5's opposing DUB UBP6 increased polyubiquitination (Crosas et al., 2006). UBP6 deletion also resulted in a reduction in sensitivity to cycloheximide, which was linked to a decrease in proteasome-mediated degradation of ubiquitin-conjugated substrates, indicating substrate polyubiquitination helps regulate global cellular ubiquitin levels crucial for accurate protein homeostasis (Hanna et al., 2003).

In humans upregulation of polyubiquitinated substrates compared to free ubiquitin is a marker of Huntington's disease (Bennett et al., 2007). Conversely clearance of protein plaques associated with Huntington's disease was facilitated by the DUB, ubiquitin-specific peptidase 14 (USP14) (Hyrskyluoto et al., 2014). The TUBE assays performed in this Chapter, while supplying data on the level of polyubiquitinated substrates, do not provide information on the status of the free pool of ubiquitin, as they preferentially bind ubiquitinated chains (Hjerpe et al., 2009). Nonetheless, reduced polyubiquitination may lead to a greater concentration of free ubiquitin in *upl3* mutants. As well as regulating ubiquitin chain length of substrates, the putative UPL3 homologues, UFD4 and HUL5, are responsible for generation of free ubiquitin chains, which can be attached wholesale to a substrate or possibly serve as storage for free monomeric ubiquitin under stress conditions (Li et al., 2007, Ravid and Hochstrasser, 2007, Kimura et al., 2009). UFD4 and HUL5 perform different roles within free chain generation: HUL5 increases release of free ubiquitin when cells are under stress, but UFD4 does not and is thought to simply maintain basal levels of free chains (Braten et al., 2012). These data indicate UPL3 could be acting in an E3 ligase capacity, possibly by the formation of free ubiquitin chains, or as an E4 ligase to extend pre-existing substrate-fused ubiquitin chains, or both depending on the cellular need.

Lower polyubiquitination levels in *upl3* and *upl3 upl4* plants correlated with reduced expression of *PR* genes after SA induction. However *WRKY* genes that are also dependent on SA showed differing expression patterns between several assays, from lower than wild type induction in Figure 4.8 to wild type levels in Figures 4.9 and 4.11, indicating that there may be a difference in WRKY expression related to plant age or the time of harvesting tissue. This expression pattern is unusual as the vast majority of SA-dependent genes require NPR1 to induce expression changes, including *PR* and *WRKY* genes (Wang et al., 2006). Thus, reduced sensitivity to SA is expected to repress expression of both gene

classes. Unlike the previously discussed *ube4-2* mutant, *upl* mutants did not show proteasome-independent transcription, indicating UPLs are not functioning as E4 ligases involved in the coordination of NPR1 transcriptional activity.

The restricted *PR* expression in *upl3* translated into reduced resistance to disease both basally (Figure. 4.14) and after immune priming with SA (Figure. 4.12). Although *upl4* mutants had no obvious differences in SA-induced genes compared to the wild type, they were more susceptible to disease although not much as *upl3* (Figure 4.12), hinting that UPL4 may play a role in disease resistance that is different from conventional SAR. Indeed the *upl3 upl4* double mutant had a similar SA-induced transcriptional profile to *upl3*, but the *upl3 upl4* double mutants were extremely susceptible to the virulent pathogen *Psm* ES4326, even more so than *upl3* and the negative control *npr1-1* (Figure. 4.14). Although the vast majority of SA-dependent genes are regulated by NPR1, there is also a parallel NPR1-independent pathway that can confer disease resistance (Zhang et al., 2003a, Bowling et al., 1997, Uquillas et al., 2004, Shah et al., 2001). The extremely high level of disease susceptibility in the *upl3 upl4* double mutant suggests that both the NPR1-dependent and NPR1-independent defences are abolished, which is highly unusual and suggests that UPL3 and/or UPL4 can act in concert with and parallel to NPR1. It could be possible that UPL3 is required for *PR* gene expression mediated by NPR1, but that UPL4 is involved in an NPR1-independent capacity, as *upl4* mutants displayed wild type transcriptional marker responses to SA induction, but were more susceptible to virulent *Psm* ES4326 after SA induction than wild type. These data indicate that UPL4 acts on an as yet unknown subset of the SA-regulated transcriptome that mediates defence against *Psm* ES4326. Therefore UPL3 and UPL4 could target different pathways within plant defences.

To further understand the role of UPL3 in transcription, RNA-seq was carried out on wild type and *upl3-1* plants sprayed with SA or water. SA induction led to 1918 genes with a two-fold or greater change in expression within wild type (Figure 4.18). Differential expression was severely curtailed in *upl3* with only 658 genes in the same range of fold change (≥2) (Figure 4.18). Heat mapping of genes that were found to be expressed in both genotypes revealed that for the majority of expression changes in *upl3* were not as pronounced as in wild type (Figure 4.19), indicating *upl3* is unable to correctly orchestrate large scale changes in expression levels rather than simply not activating a sub-set of SA-dependent genes. Reminiscent of this auxiliary role of UPL3, the transcriptional regulator WRKY18 similarly enhanced SA-responsive gene expression, as knockout of WRKY18 dampened the expression level of NPR1-dependent genes (Wang et al., 2006). Comparison of UPL3-, BTH- and NPR1-dependent genes revealed nearly all UPL3 dependent genes that overlapped with BTH-regulated genes, were also dependent on NPR1 (Figure 4.20), suggesting UPL3 attenuates NPR1 target gene expression. Of the genes which showed the highest difference in fold change after SA treatment between wild type and *upl3*, SA-induced genes that were down-regulated in *upl3* exhibited a high overrepresentation of the WRKY-binding W-box in their promoters (Figure 4.21 & 4.22). These data imply that UPL3 enhances NPR1-mediated transcription by the targeting and ubiquitination of immune-related WRKYs. Indeed, UPL3 has already been shown to target the immune-related WRKY53 for degradation (Miao and Zentgraf, 2010). Moreover, the recent discovery of WRKY70 as a repressor of NPR1-dependent genes provides a clue to how UPL3 could amplify SA-dependent transcription. Sumolyation of NPR1 is required to relieve WRKY70 repression and produce transcription competent NPR1 (Saleh et al., 2015). Degradation of WRKY70 mediated by UPL3 could be required to provide full defence induction via the destruction of this repressor. This could occur either by ubiquitinating WRKY70 after it has

dissociated from NPR1 or by sumolyation events that recruit UPL3 to the NPR1-WRKY70 complex after which WRKY70 removal is directly mediated by the UPS. WRKY70 along with WRKY54 also act to suppress SA biosynthesis (Wang et al., 2006). UPL3-mediated ubiquitination of these proteins could be required in order to fully up-regulate SA synthesis in early signalling stages and may also explain why basal defences in *upl3-1* mutants were compromised (Figure 4.14). UPL3 could also target WRKY58, which acts as a repressor of SA signalling (Wang et al., 2006). Additionally, UPL3 could target other repressors of SA signalling that are not WRKYs, such as NIMIN proteins. NIMINs bind to NPR1 and are able to form a complex containing TGA factors, over expression of NIMINs curtailed PR gene expression, knockout of NIMINs had the opposing effect, which is highly indicative of NIMINs acting as NPR1-mediated transcription repressors (Weigel et al., 2005, Weigel et al., 2001).The NPR1 repressor SNI1 is involved in maintaining chromatin integrity (Fu and Dong, 2013, Yan et al., 2013, Durrant et al., 2007), as excessive transcription can increase the likelihood of DNA damage. Removal of SNI1 by UPL3 could allow rapid remodelling of the chromatin to make it more suitable for transcription.

UPL3 was also found to be involved in the regulation of SA-repressed genes containing an E-box (CANNTG) promoter motif. E-boxes are highly variable; alteration of the two core NN residues can provide greater specifity for the binding of particular TFs (Yutzey and Konieczny, 1992). Promoter analysis of the RNA-seq data revealed that the E-box variants CACATG, known as the Hormone Up at Dawn motif (HUD) (Michael et al., 2008), and the G-box (CACGTG) were enriched in genes up-regulated in *upl3* (Figures 4.22 & 4.23), indicating that UPL3 is required to suppress these genes in SA-treated wild type. G-boxes are bound with high affinity by MYC2 which is involved in regulation of the JA signalling pathway (Dombrecht et al., 2007) and operates antagonistically with the SA pathway (Pieterse et al., 2009). Indeed, JA-responsive genes are present amongst the genes up-

regulated in *upl3* compared to wild type (Supplemental 4.1) hinting that UPL3 is required to suppress JA signalling in response to SA induction by targeting MYC transcription factors. The HUD motif is also over-represented in JA-dependent genes regulated by MYC2. However the role of the HUD motif in this context is unclear as it was found to have a lower binding affinity for MYC2 than the G-box (Dombrecht et al., 2007). A further role in gene repression for UPL3 can be suggested as the HUD motif was discovered to be involved circadian responses regulating cell growth and elongation (Michael et al., 2008). In accordance, down-regulation of 'house-keeping' genes such as those responsible for growth is observed after SA treatment, as the plant prioritizes defence responses (Scheideler et al., 2002). The high incidence of the HUD motif in those genes up-regulated in the absence of UPL3 indicates the switch in gene expression priority appears incomplete in *upl3* mutants.

From the data provided here, we conclude UPL3 is a novel and essential regulator of SA-dependent defences, facilitating the large-scale gene expression changes mediated by NPR1. Future work on UPL3 should focus on the discovery of proteins directly interacting with UPL3, which would provide key information in how this ubiquitin ligase plays such an important role in the orchestration of plant immunity.

Chapter 5: Investigation into the role of two E4 ligases in transcription initiation in yeast

5.1. Introduction

As detailed in the General Introduction, yeast are readily able to produce all amino acids, the manufacturing of which is closely linked to the levels of amino acids in the growth media. Removal of an amino acid leads to increased synthesis of all amino acids by a process known as General Amino Acid Control (GAAC) (Hinnebusch, 2005), which is regulated by the transcription factor GCN4 that requires proteasome-mediated degradation in order to correctly activate GAAC-related transcription (Lipford et al., 2005). When GCN4 activates a round of transcription it is marked for degradation by phosphorylation by the kinase SRB10 located on the C-terminal domain of RNA polymerase II. Phosphorylated GCN4 is then ubiquitinated by the E3 ligase CDC4 and degraded by the proteasome (Lipford et al., 2005). This mechanism could provide an excellent model in which to discern the role of E4 ligases in transcription initiation.

The first E4 ligase, UFD2, was described in yeast, where it was shown to enhance ubiquitin chain elongation, including the K48-linked ubiquitin chain, which is the canonical linkage to signal for the degradation of a substrate by the 26S proteasome (Koegl et al., 1999, Saeki et al., 2004). UFD2 functions similar to E3 ligases by forming a bridge between the substrate and the E2 conjugating enzyme, UBC13, that binds via its C-terminal U-box domain and allosterically activates transfer of ubiquitin to the substrate (Smalle and Vierstra, 2004, Tu et al., 2007). U-boxes are structurally similar to the RING (Really Interesting New Gene) domain found in many E3 ligases but use electrostatic interactions to stabilise a RING-like finger instead of zinc chelation used by actual RING domains (Ohi et

al., 2003). Although UFD2 can directly bind an E2 enzyme, an E3 enzyme is required for the reaction to proceed, even if a polyubiquitinated substrate is provided (Tu et al., 2007, Koegl et al., 1999). The enhancement of ubiquitin chain formation by UFD2 could work sequentially to or in direct partnership with the E3 ligase, which could provide increased specificity to a particular substrate required for the reaction (Tu et al., 2007). Indeed, UFD2 can work in partnership with the E3/E4 enzyme UFD4 (Koegl et al., 1999, Tu et al., 2007), UFD4 has been shown to enhance the processivity of another E3 ligase, UBR1 (Hwang et al., 2010). Substrates polyubiquitinated by UFD2 are passed to RAD23, a shuttle protein responsible for escorting substrates to the proteasome. The handover between UFD2 and RAD23 is made possible by interaction of the UFD2 N-terminal variable region that binds to the ubiquitin-like (UBL) domain on the N-terminus of RAD23. RAD23 is then thought to bind to the polyubiquitinated substrate via its ubiquitin-associated (UBA) domains. UFD2 also contains a core UFD2 domain, which contains domains similar to the Armadillo motifs found in importin-α, which is used by UFD2 during interaction with other proteins including the co-factor CDC48 (Tu et al., 2007, Hanzelmann et al., 2010, Azevedo et al., 2001). The UFD2-RAD23 complex is likely separated by CDC48 allowing the UBL domain of RAD23 to bind the proteasome subunit RPN1, which acts as a docking site for ubiquitin processing factors. The binding of CDC48 to UFD2 is critical to its function; as mutation of the C-terminus of CDC48, which provides the UFD2 binding site, produces an effect that phenocopies the *ufd2Δ* mutant (Kim et al., 2004, Hanzelmann et al., 2010, Baek et al., 2011, Bohm et al., 2011) This data suggests a model in which UFD2 catalyses enhanced chain elongation on ubiquitinated substrates, the shuttle protein RAD23 then guides the complex to the proteasome where CDC48 is required to remove UFD2 from RAD23, allowing substrate degradation. This mechanism would provide an efficient and reliable system to orderly degrade unwanted proteins.

Another yeast E4 ligase, HUL5, does not contain the U-box domain and instead uses a HECT (Homology to E6AP C Terminus) domain (Crosas et al., 2006), which, unlike the RING/U-box, contains a unique cysteine that allows the activated ubiquitin-E2 thioester to be transferred to the HECT domain before addition to the substrate (Ravid and Hochstrasser, 2008). HUL5 is a proteasome-associated protein via a salt labile interaction (Leggett et al., 2002), where it acts to increase polyubiquitination on a wide range of substrates (Crosas et al., 2006). HUL5 is required to correctly degrade proteins during the heat shock response (Fang et al., 2011) and is responsible for the generation of free chains during stress events, including heat shock (Braten et al., 2012). This indicates that during times of stress, which can produce large amounts of misfolded, damaged and unwanted proteins, enhancement of the ubiquitin chain by HUL5 helps to promote rapid degradation. Proteasome subunit RPN2 provides the docking site for HUL5, where it is brought into proximity with and works antagonistically to the deubiquitinase (DUB) UBP6, which binds the proteasome RPN1 subunit (Crosas et al., 2006, Leggett et al., 2002). The opposing actions of ubiquitin chain lengthening and reduction provided by HUL5 and UBP6, respectively, could provide the proteasome with flexible decision power to degrade or excuse ubiquitinated substrates.

This chapter aims to investigate if the E4 ligases, UFD2 and HUL5, play a role in regulating processive ubiquitination of GCN4 to modulate GAAC-related transcriptional responses.

5.2. Results

5.2.1. Deletion of UFD2 results in increased basal levels of GCN4-13Myc

Wild type yeast cell are able to synthesize all essential amino acids when there are insufficient amounts in the surrounding environment. Lab yeast strains have selected mutations within the synthesis pathways, so they are auxotrophic for specific amino acids. The yeast are grown in synthetic minimal media (SMM) containing all the amino acids the strains are auxotrophic for. Induction of the GAAC can be induced by switching of this media for SMM without leucine (Lipford et al., 2005). As yeast can only detect that an amino acid is missing, but not which one, the GAAC pathway is activated to produce all amino acids, leading to approximately 500 genes being upregulated (Hinnebusch, 2005). To investigate the role of E4 ligases within the GAAC response we obtained a deletion mutant of the E4 ligase UFD2 (Koegl et al., 1999) from the Euroscarf collection (Euroscarf, 2015). As deletion of an E4 ligase can alter the rate of degradation of target substrates (Koegl et al., 1999, Crosas et al., 2006); we epitope-tagged GCN4 with 13Myc in both the wild type and *ufd2Δ* background. Thus, proteins levels of GCN4 could be monitored for differences between the genotypes with and without starvation induction via the removal of leucine from the media. Levels of GCN4-13Myc were undetectable in the wild type background before induction, while after induction levels of GCN4-13Myc increased (Figure 5.1). Although leucine starved *ufd2Δ* cells had similar levels of GCN4-13Myc as wild type, they displayed increased basal levels of GCN4-13Myc (Figure 5.1), indicating that UFD2 is involved in GCN4 regulation.

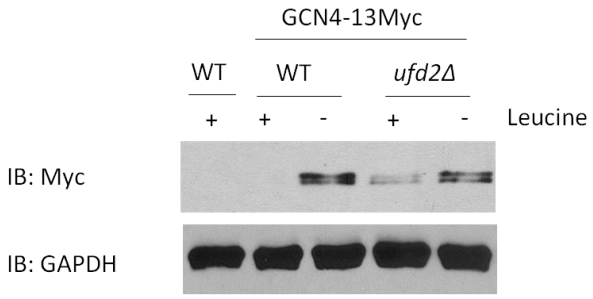

Figure 5.1: Deletion of *UFD2* results in increased basal levels of GCN4-13Myc.

Cells were grown in SMM with leucine to mid-log before refreshing the media with (+) or without (-) leucine for 2 hours. Protein extracts were analysed with SDS-PAGE and western blotting by probing for α-Myc and α-GAPDH as a loading control.

5.2.2. Deletion of UFD2 fails to prevent turnover of GCN4-13-Myc

As GCN4 requires degradation to facilitate transcription, we blocked protein synthesis with the translation inhibitor cycloheximide, which as expected led to the depletion of GCN4-13Myc protein (Figure 5.2). Because UFD2 extends ubiquitin chains to increase the potential for turnover of a protein by creating a higher affinity for proteasome binding, *ufd2Δ* mutants may exhibit an altered degradation rate of GCN4. Therefore we investigated the stability of GCN4-13-Myc in leucine-starved wild type and *ufd2Δ* cells in a cycloheximide chase assay. In wild type cells, GCN4-13Myc levels gradually decreased over the course of 120 minutes post application of cycloheximide (Figure 5.3). Unexpectedly, however, in *ufd2Δ* cells GCN4-13Myc levels also decreased steadily and even showed a slightly faster clearance of GCN4-13Myc protein at 60 minutes post cycloheximide treatment than wild type. These data suggest that UFD2 is not a major factor in the polyubiquitination and subsequent turnover of GCN4 in leucine-starved cells.

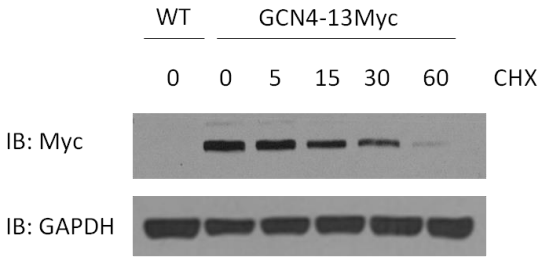

Figure 5.2: GCN4-13Myc protein is degraded during the GAAC response.

Cells were grown to mid-log phase in SMM with leucine. Media was switch to SMM –Leucine for 1 hour, then 100 μM cycloheximide was added. Samples were then collected at indicated time points. Protein extracts were analysed with SDS-PAGE and western blotting by probing with α-Myc and α-GAPDH as a loading control.

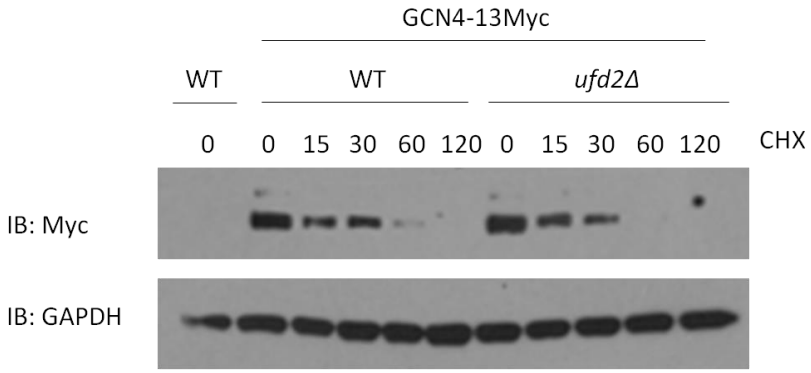

Figure 5.3: The E4 ligase mutant *ufd2Δ* does not prevent turnover of GCN4-13Myc in starvation conditions.

Cells were grown to mid-log phase in SMM with leucine. Media was switch to SMM –Leucine for 1 hour, then 100 μM cycloheximide was added. Samples were then collected at indicated time points. Protein extracts were analysed with SDS-PAGE and western blotting by probing with α-Myc and α-GAPDH as a loading control.

5.2.3. Deletion of HUL5 but not UFD2 increases basal levels of GCN4 target genes

To assess transcriptional responses of the GAAC pathway in our experimental system we performed a time course to monitor the expression of several key marker genes post starvation. Most marker genes peaked in expression within 30 minutes of leucine starvation and expression was maintained at levels higher than before induction thereafter (Figure 5.4). Thus, subsequent gene expression experiments in this chapter were performed at 60 minutes post starvation.

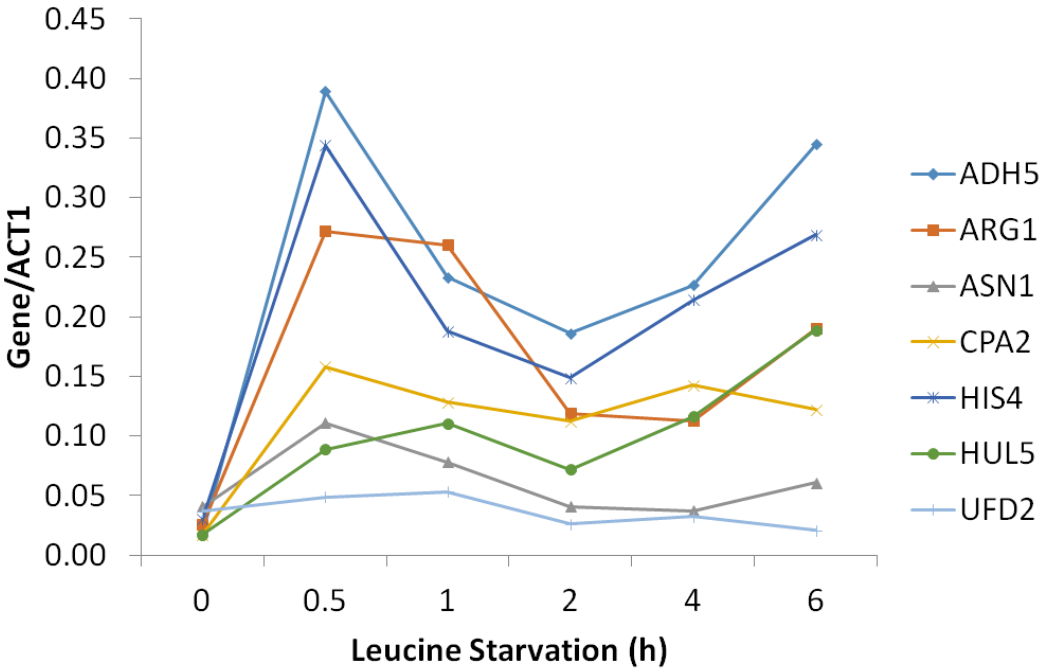

Figure 5.4: Removal of leucine from the media leads to activation of genes in the amino acid synthesis pathway.

Wild type yeast were grown in Synthetic Minimal Media (SMM) +Leucine until mid-log phase. Yeast was then switched to SMM –Leucine and samples taken at indicated times. Samples were analysed by qPCR and gene expression normalised to *ACT1*.

Although *ufd2Δ* has little effect on GCN4 stability we investigated if the GAAC had a compromised response in the *ufd2Δ* background as this mutation may have an effect on the intrinsic transcriptional activity of GCN4 or on other regulators of the GAAC. We also included another E4 ligase deletion mutant, *hul5Δ*, which had been shown to stabilise GCN4 turnover (Crosas et al., 2006). Wild type and mutants were grown in SMM to mid-log phase OD 0.5-1.0, before being switched to SMM lacking leucine. Wild type yeast had low expression of marker genes in the presence of leucine, while after GAAC induction gene expression dramatically increased (Figure 5.5). Whereas deletion of *UFD2* resulted in a largely wild type response, *hul5Δ* mutants displayed increased basal expression across all genes and reduced expression of most genes upon leucine starvation. These results indicate that HUL5 is required to prevent unwanted activation of GAAC-induced genes and may also be involved in their activation.

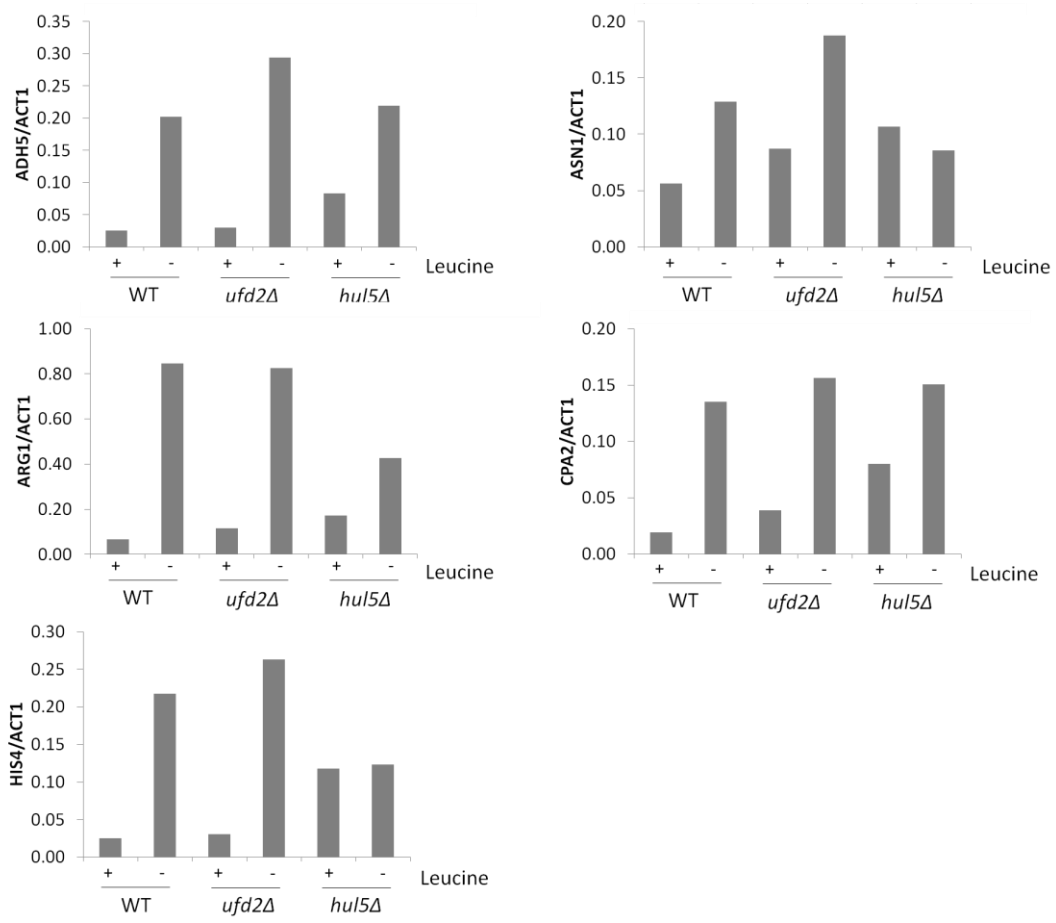

Figure 5.5: Deletion of HUL5 leads to increased basal transcription and reduced the GAAC response.

Cells were grown to mid-log phase in synthetic minimal media (SMM) with leucine before being switched to a media with (+, control) or without (-, induced) leucine for 2 hours. Samples were analysed by qPCR and gene expression normalised to *ACT1*.

As *hul5Δ* showed compromised transcription regulation, we next decided to investigate if control of transcription initiation within the E4 ligase mutants was dependent on GCN4 degradation by the proteasome. In this experiment the proteasome was inhibited by the addition of MG132, however yeast cells are generally resistant to this chemical as they are able to remove it from the cell via the efflux pump pleiotropic drug resistance 5 (PDR5). Mutation of this gene may have unknown effects on cell growth and metabolism

(Liu et al., 2007). Thus, to allow the use of MG132 in this experiment we followed a protocol in which the SMM had the nitrogen source changed from ammonium sulphate to 1 % proline and 0.003 % SDS was added to cause mild permeability of the cell membrane, which allows MG132 to be taken up more efficiently by the cell (Liu et al., 2007). Cells were grown to mid-log phase then pre-incubated with 100 µM MG132 or vehicle (DMSO), before switching the media to SMM –Leucine with MG132 or vehicle for one hour. The results of the experiment was analysed via qPCR and the difference in gene expression between samples with and without MG132 was calculated as percentage of gene expression suppressed by MG132 treatment. In wild type, target genes had a reduction in expression of 85 % or more for all genes except *ASN1*. The E4 ligase mutants displayed comparable reductions in target gene expression except for *ASN1* for which the mutants had increased suppression. These results illustrate that *ufd2Δ* and *hul5Δ* are unlikely to be involved in the regulation of GCN4-degradation during amino acid starvation as they do not show any proteasome-independent GAAC responses.

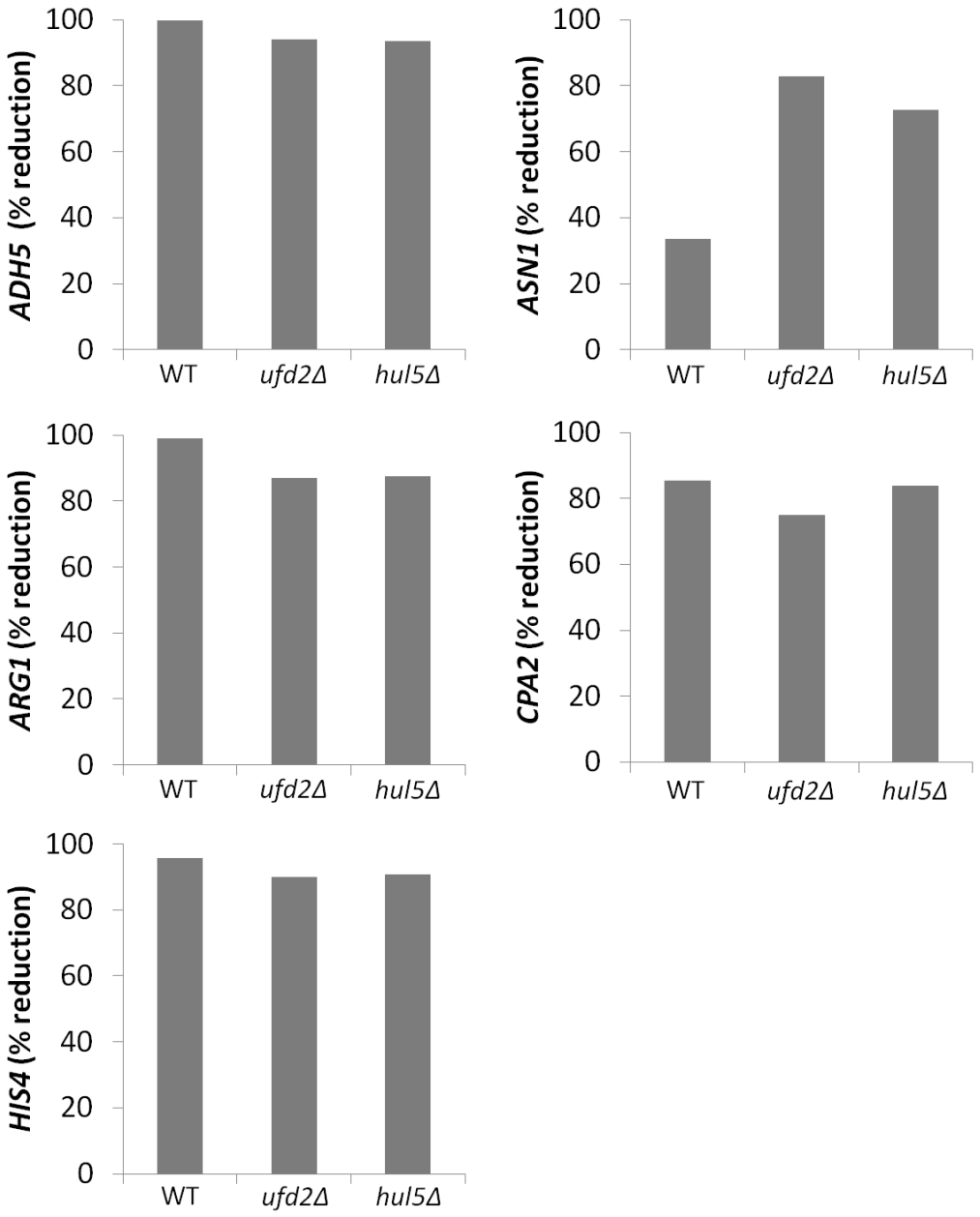

Figure 5.6: E4 ligase mutants have a similar response to proteasome inhibition as the wild type.

Cells were grown in SMM with 1 % proline as the nitrogen source, leucine and 0.003 % SDS, to mid-log phase, pre-incubated with MG132 or vehicle (DMSO) for 30 minutes before refreshing the media supplemented with (+) or without (-) leucine for 2 hours. Samples were analysed via qPCR and gene expression normalised to *ACT1*. Data was expressed as percentage reduction in gene expression observed in MG132-treated cells compared to vehicle-treated cells.

5.3. Discussion

When yeast is grown in a media that lacks an amino acid, it responds by upregulating the production of all amino acids (Hinnebusch, 2005). This leads to an increase in transcription of over 500 genes orchestrated by the master transcription activator GCN4 (Natarajan et al., 2001). In order to maintain the expression of genes induced by amino acid starvation GCN4 requires ubiquitination and proteasome-mediated degradation (Lipford et al., 2005). Using this inducible system we investigated the role of two E4 ligases, UFD2 and HUL5 on transcription induced by unstable GCN4. We discovered that deletion of UFD2 had minor effects on GCN4 stability and did not alter GAAC transcriptional regulation. However, mutation of HUL5, which has been reported to prevent GCN4 turnover (Crosas et al., 2006), resulted in increased basal transcription, indicating this E4 is required for correct regulation of GCN4 and warrants further study.

Depending on cellular amino acid homeostasis, the GAAC master regulator GCN4 is targeted for degradation by different methods. In basal conditions GCN4 is rapidly phosphorylated by the kinase phosphate metabolism 85 (PHO85) which signals for ubiquitination via the F-box protein CDC4 (Irniger and Braus, 2003). Upon starvation induction PHO85 is down-regulated and GCN4 is able to accumulate and activate transcription, at which point it is phosphorylated by SRB10 and then ubiquitinated, again by CDC4 (Lipford et al., 2005). Deletion of UFD2 led to greater stability of basal GCN4 but a minor increase in turnover after leucine starvation (Figures 5.1 & 5.2) and did not have any effect on the transcription of target genes (Figure 5.5). A hypothesis that arises from this is that UFD2 is required for the correct regulation of GCN4 degradation in basal conditions. However, as an increase in associated transcription was not seen under basal conditions, it possible that despite its elevated levels, GCN4 was not transcriptionally active. The minor

starvation-induced increase in GCN4 turnover in *ufd2Δ* cells could be too subtle to have an effect on transcription. By contrast, basal increases in transcription were seen in *hul5Δ* (Figure 5.5), which corresponds to HUL5's recently reported role in the degradation of GCN4 in uninduced conditions (Crosas et al., 2006). Moreover, deletion of *HUL5* reduced starvation-induced transcriptional responses (Figure 5.5), suggesting that HUL5 plays a cotranscriptional role in GAAC responses.

In chapter 3 we saw that the deletion of an E4 ligase can lead to a disconnection of TF activity and proteasome-mediated degradation, indicating initial ubiquitination produced a highly active TF and that its subsequent destruction was required to limit that activity. In this chapter neither *ufd2Δ* nor *hul5Δ* presented a proteasome-independent transcription phenotype. These data indicate that neither of these E4 ligases are required for starvation-induced GCN4 turnover. Both UFD2 and HUL5 are non-essential for survival under normal conditions, but are required for appropriate responses to cellular stress (Koegl et al., 1999, Fang et al., 2011, Braten et al., 2012). Because HUL5 is also responsible for generating free ubiquitin chains during cellular stress (Braten et al., 2012), HUL5 could cotranscriptionally regulate GAAC marker genes in an GCN4-independent manner. Alternatively, HUL5 could indirectly regulate the turnover of GCN4 through ubiquitination of other factors or by proteasome-independent non-Lys48 ubiquitination of GCN4. Unfortunately *hul5Δ* cells grew very poorly in the SMM media used making comparisons to wild type and *ufd2Δ* in protein experiments difficult. This technical difficulty needs to be overcome to investigate how *hul5Δ* alters GCN4 stability and GAAC gene transcription. A key experiment that could not be performed due to time constraints on this project, would be visualisation of the ubiquitination status of GCN4 in the wild type and mutants, which could be performed using the TUBE protocol (Hjerpe et al., 2009). This would allow a link to be drawn between gene expression and polyubiquitination status, as seen in chapters three

and four. As *ufd2Δ* has no effect on GCN4-mediated transcription, further investigation should be directed towards HUL5 in the GAAC response.

As mentioned above HUL5 acts in opposition to UBP6, it would beneficial to also include *ubp6Δ* into experiments for further investigation as monitoring the antagonistic actions of both an E4 ligase and a DUB on a model substrate such as GCN4 could elucidate how the dynamics of ubiquitination alters the activity of a transcription factor.

Chapter 6: General Discussion

This PhD project set out to investigate if E4 ligases play a role in the regulation of transcription initiation by controlling TF activity. To achieve this, the project looked at two canonical E4 ubiquitin ligases in yeast and how they altered the GAAC response via modulation of the TF GCN4. Using sequence alignments to the E4 ligases found within yeast we identified putative homologues in *Arabidopsis* and tested their function in activation of SA-responsive immune gene expression.

In chapter 3 we found that UBE4, a homolog of the yeast UFD2, acted as a repressor of SA-dependent transcription. Knockout of UBE4 led to stabilisation of the SA-responsive master immune coactivator NPR1. Stabilisation of NPR1 in *ube4-2* knockout mutants was due to reduced levels of polyubiquitination of NPR1 and was associated with increased transcription of NPR1 target genes. The phenotype produced by mutation of UBE4 differed from previous experiments that stabilized NPR1. Contrary to mutation of NPR1's phospho-degron, mutation of the E3 ligase CUL3, and inhibition of proteasome activity, all of which stabilized NPR1 and significantly reduced NPR1 target gene transcription (Spoel et al., 2009), mutation of UBE4 resulted in a highly transcriptionally competent NPR1. These findings have important implications for how E3 and E4 ligases potentially control transcription through processive ubiquitination of NPR1. Our data suggest that CUL3-mediated ubiquitination initially activates NPR1 and that subsequent processive polyubiquitination by UBE4 limits this activity by targeting NPR1 for proteasome-mediated degradation. This hypothesis is supported by the observation that *ube4-2* plants were able to maintain high levels of SA-induced NPR1 target gene expression in presence of proteasome inhibitor.

In mammalian cell systems E4 ligases have been studied in the regulation of the tumour suppressor p53. Like NPR1, p53 is constantly transcribed and translated in unstressed cells but basal levels are kept low through constant ubiquitination and degradation. In times of genotoxic stress, however, ubiquitination of p53 is suppressed, leading to stabilisation of the protein and rapid upregulation of p53 target genes (Hock and Vousden, 2014). Ubiquitination of p53 is regulated by over a dozen E3 ligases and five potential E4 ligases (Love and Grossman, 2012, Pant and Lozano, 2014). Interestingly, one of the key E3 ligases involved in p53 regulation, MDM2, is only able to monoubiquitinate its substrates and relies on E4 ligases to promote further polyubiquitination either by increasing the efficacy of p53 ubiquitination in co-operation with an E3, or through direct intrinsic E4 ubiquitin ligase activity (Pant and Lozano, 2014, Love and Grossman, 2012). The mammalian UBE4 is likely a member of the former group and extends ubiquitin chains on p53 in the presence of MDM2. UBE4 activity on p53 is only found in the presence of MDM2 and is unable to alter p53 ubiquitination status in absence of MDM2 (Wu et al., 2011). UBE4 shares the responsibility to polyubiquitinate p53 with two other E4 ligases: Gankyrin, which increases MDM2 ligase activity towards p53 and the proteasome (Higashitsuji et al., 2005), and Yin Yang1, which promotes ubiquitination through enhancing MDM2/p53 interaction (Sui et al., 2004). It is currently unknown why p53 has so many E4 ligases supporting polyubiquitination, or if they serve distinct functions in different circumstances: For example, monoubiquitination of p53 by MDM2 can lead to transfer of p53 from the nucleus to the cytoplasm, where P300 and its paralog, CREB-binding protein (CBP) act as E4 ligases, extending the p53 monoubiquitination mark into a polyubiquitin chain. Cellular localisation is important for the function performed by P300 and CBP as within the nucleus they function as histone acetyltransferases, however within the cytoplasm they function through

an, as yet ill-defined, intrinsic ubiquitin ligase domain (Grossman et al., 2003, Grossman et al., 1998, Shi et al., 2009).

In yeast the RING-type E3 ligase UBR1 acts to ubiquitinate substrates of the N-end rule pathway. Its function is enhanced in the presence of the HECT-type ubiquitin ligase UFD4, which normally functions in the UFD pathway (Hwang et al., 2010). Conversely the opposite is true with UFD4 function aided by UBR1 (Hwang et al., 2010). Not only does this unite what were considered two independent ubiquitination pathways, it also illustrates that the function of a ubiquitin ligase is interchangeable between E3 and E4 enzymes depending on the context. In case of the *Arabidopsis* UBE4 ligase, it would be interesting to know if its polyubiquitination function is also dependent only on the presence of a (mono)ubiquitin mark on the substrate or if the presence of an adaptor or cofactor is required to provide specificity to UBE4 activity. As shown with the mammalian p53/MDM2 system and yeast UBR1/UFD4, it is possible that the presence of an E3 ligase at the target substrate is able to recruit UBE4 to perform polyubiquitination. In *Arabidopsis* NPR1/UBE4 interaction may be mediated by the presence of the CUL3 ligase. The fact that p53 protein levels are regulated by a multitude of E3 and E4 ligases hints that UBE4 may not be the only E4 to influence NPR1 degradation. However, to date NPR1 has only been show to interact with the CUL3 ligase mediated by the two F-box adaptor proteins NPR3 and NPR4 (Fu et al., 2012), and as UBE4 seems to influence both basal and SA-induced gene expression via NPR1, the action of further E4 ligases may be unnecessary. This does not mean that UBE4 does not exert influence over other pathways within *Arabidopsis*. Indeed, the growth defects seen in *ube4-2* hint that it may be involved in pathways influencing development and morphology. If indeed the action of UBE4 is mediated by the presence of an E3, coimmunoprecipitation of UBE4 and cognate E3s combined with mass spectrometry may reveal new potential UBE4 targets.

In chapter five we investigated the role of two canonical E4 ligases in yeast: the RING-like U-box protein UFD2, the first described E4 ligase (Koegl et al., 1999), and the HECT-domain containing HUL5, which is bound to the proteasome and acts in opposition to the deubiquitinase UBP6 (Leggett et al., 2002, Crosas et al., 2006). Deletion of UFD2 led to the stabilisation of the general amino acid control master regulator GCN4 at basal levels of transcription but mildly increased degradation after induction following removal of leucine from the system. Similar to NPR1, GCN4 requires proteasome mediated turnover (Lipford et al., 2005). However, previous methods have either blocked initial GCN4 ubiquitination or chemically inhibited the proteasome to prevent highly ubiquitinated GCN4 from being degraded (Lipford et al., 2005). By knocking out the E4 ligases UFD2 and HUL5 we hoped to investigate if processive ubiquitination altered GCN4 activity in yeast similar to our findings for NPR1 in plants. However, *ufd2Δ* cells did not dramatically differ from wild type in GCN4 turnover rate and transcriptional output of GCN4 target genes, suggesting UFD2 does not play a major role in the polyubiquitination of GCN4. Deletion of HUL5, however, produced increased levels of basal GCN4 target gene transcription and also decreased starvation-induced gene expression. Unfortunately, it was not possible to investigate GCN4 protein levels further as slow growth of *hul5Δ* cells made experimental comparison to wild type difficult. Nonetheless, we speculate that HUL5 may play an important role in controlling GCN4 activity through processive ubiquitination in a similar manner as *Arabidopsis* UBE4 controlled NPR1 activity. As yeast HUL5 is bound to the proteasome, its opposition to the deubiquitinase UBP6 could act as a timer for GCN4 activity and functionality (Leggett et al., 2002, Crosas et al., 2006). On the other hand, *Arabidopsis* UBE4 is expected to act in complex with the shuttle protein Rad23 to supply polyubiquitinated NPR1 to the proteasome (Tu et al., 2007, Hanzelmann et al., 2010). The differing localisations between HUL5 and UBE4 may suit different purposes in transcription (co)factor regulation or both E4

ligases may act cooperatively to promote transcription factor ubiquitination as seen in p53/MDM2/UBE4. Hence, an aspect of our investigation that was not possible due to time constraints, would be to investigate if a yeast *ufd2Δ hul5Δ* double mutant displays increased effects on GCN4 protein degradation and corresponding transcriptional output.

Chapter four was dedicated to using the homology to yeast HUL5 to find further putative E4 ligases in *Arabidopsis*. This approach yielded the UPL protein family, of which we investigated four members that fall into two classes: UPL3 and UPL4 on the one hand, and UPL6 and UPL7 on the other. Interestingly, this revealed UPL3 to be a major regulator of both total cellular as well as substrate-specific ubiquitination events. More importantly, reduced cellular polyubiquitination in *upl3* knockout mutants correlated with compromised SA-dependent gene expression, leading to enhanced disease susceptibility that was further increased in *upl3 upl4* double mutants, suggesting some functional redundancy between these proteins.

The discovery of UPL3 as a novel ubiquitin ligase in the regulation of SA-responsive gene expression is significant as most ubiquitin ligases known to date function upstream of SA and NPR1. An early screen for mutations leading to SA-mediated autoimmune phenotypes identified the *cpr1* mutant (Bowling et al., 1994). Importantly, protein levels of the NLR receptors SNC1 and RPS2 were inversely correlated with CPR1 activity, and loss-of-function mutations in *SNC1* largely suppressed the autoimmune phenotype of mutant *cpr1* plants. Cloning of *CPR1* revealed it encodes an F-box protein, suggesting it controls the abundance of NLR receptors by targeting them for proteasome-mediated degradation. Indeed, CPR1 directly interacted with SNC1 and RPS2, and in case of SNC1 this appeared to lead to its polyubiquitination and degradation by the proteasome (Cheng et al., 2011, Gou et al., 2012). NLR receptor signaling probably involves other CRL1 ubiquitin ligases as well

but with distinct functions. Rather than eliciting autoimmunity, silencing of the F-box protein (Avr9/Cf-9 induced F-box 1) ACIF1 in tobacco and tomato compromised NLR receptor-mediated PCD and immunity (van den Burg et al., 2008). ACIF1 interacted with other CRL1 subunits, suggesting it can form a functional ubiquitin ligase but its direct targets remain unknown. The proteins plant U-box 12 (PUB12) and PUB13 are involved in the regulation of the flagellin receptor FLS2. PUB12 and PUB13 recruitment to FLS2 is dependent on phosphorylation by *Botrytis* induced kinase 1 (BIK1), a process that is enhanced in the presence of flagellin and BRI1-associated kinase 1 (BAK1). Deletion of the PUB12/13 led to increased responses to flagellin and disease resistance, suggesting ubiquitination of flagellin sensitive 2 (FLS2) is used to negatively regulate defence responses (Lu et al., 2011) . The RING domain containing RPM-interacting proteins RIN2/RIN3 proteins interact with the NLR receptor Resistance to *P. Syringae* pv. *Maculicola* 1 (RPM1), which confirms resistance to *P. syringae* carrying the AvrRPM1 effector. Upon onset of HR, RPM1 levels reduce in a proteasome-dependent manner. However RIN2/RIN3 do not directly ubiquitinate RPM1 but are functional ligases, suggesting they target an RPM1-associated protein while another E3 is responsible for directly targeting RPM1 to the proteasome (Boyes et al., 1998, Kawasaki et al., 2005). Other RING proteins have also been implicated in regulation of pathogen defense. Increased SA accumulation can be realized through mutation of the RING E3 ligase benzoic acid hypersensitive 1-Dominant (BAH1). Increased SA levels were both dependent on ICS1, a key biosynthetic enzyme in SA production, and independent from ICS1, potentially operating through alternative SA pathways or via the redundant ICS2, however the mechanism leading to mis-regulation of gene expression is not understood (Yaeno and Iba, 2008). In the pepper *Capiscum annuum* silencing of the E3 ligase RING1 reduced levels of SA, HR cell death and *PR1* gene expression, leading to associated disease susceptibility. Conversely, over-expression of *CaRING1* in *Arabidopsis*

increased resistance to biotrophic pathogens. These experiments suggest that *CaRING1* is involved in defence responses through ubiquitination and regulation of cell death (Lee et al., 2011).

Although these experiments indicate that ubiquitin ligases are important in the control of pathogen responses, they do not directly help to elucidate the role of ubiquitination at the level of transcription regulation for which the only example is the previously discussed NPR1/CUL3/UBE4 relationship (Chapter 3) (Spoel et al., 2009, Fu et al., 2012). Previously published studies described the RING, CUL1/F-box and U-box E3 ligase classes, while experiments performed in this thesis show the first HECT domain E3 ligase involved in SA-dependent disease responses. Direct application of SA in our experiments bypassed upstream signalling events such as pathogen receptor abundance, suggesting the phenotype shown by *upl3* is not due to mis-regulation of early signalling events. Because SA is directly perceived by transcription cofactor-receptor complexes, UPL3 likely acts cotranscriptionally. As *upl3* mutants displayed a vast reduction in SA- and NPR1-dependent gene expression, UPL3 may be acting as an auxiliary factor to promote transcription. Consistent with this hypothesis, we identified the W-box as an overrepresented element in SA-induced genes that were regulated by UPL3. The W-box binds WRKY transcription factors, many of which play critical roles in stimulating or repressing SA-responsive genes. Of particular interest in this respect is recent work that shows WRKY70 acted as a repressor of SA- and NPR1-dependent gene transcription (Saleh et al., 2015). Thus, WRKY70 is a good candidate for UPL3 action as targeted degradation of this repressor could enhance NPR1-mediated gene expression in response to SA. To test this hypothesis in future a cross between *upl3-1* and epitope-tagged WRKY70 would be required in order to experimentally test WRKY70 degradation.

6.1. Conclusions

As outlined and discussed above, the work presented in this thesis provides novel insights into the role of ubiquitin ligases in regulating transcription initiation of immune responses through the controlled proteasome-mediated degradation of key SA-responsive transcription (co)factors. A summary of the key findings is detailed below:

(1) Short ubiquitin chains activate NPR1 coactivator, leading to high levels of SA-responsive gene transcription. Subsequent polyubiquitination by the E4 ligase, UBE4, and proteasome-mediated degradation serve to limit or prevent excessive transcriptional activity of NPR1 (Chapter 3).

(2) UPL3 is a novel ubiquitin ligase essential for global cellular polyubiquitination and acts as an auxiliary factor in activating SA-responsive genes potentially by targeting WRKY repressors for proteasome-mediated degradation (Chapter 4).

(3) In *S. cerevisiae* the E4 ligase HUL5, but not UFD2, regulates amino acid starvation-induced gene expression, which requires turnover of the GCN4 transcription activator. Further research should determine if HUL5 acts cotranscriptionally by targeting GCN4 for degradation (Chapter 5).

6.2. Impact

The conclusions reached from the evidence provided in this thesis and associated literature indicate far reaching impact. In case of Chapter 3, studies on the E4 ligase activity of UBE4 illustrate TFs not only remain active after ubiquitination but the act of ubiquitination can enhance the intrinsic activity of TFs. Related to this, findings from Chapter 5 suggest that E4 ligase activity may regulate unstable transcription activator activities across the eukaryota, though with differing mechanisms of substrate targeting to the proteasome. This information could be transferred to biomedical science as several oncoproteins and developmental transcription (co)activators are unstable and their turnover is required for the activation of their target genes. Like the mammalian oncoprotein p53, these (co)activators may be polyubiquitinated by the mammalian homolog of UBE4/UFD2. In *Arabidopsis* UBE4 could have other targets than NPR1 and their isolation could provide clues to which further TFs are regulated, at least in part, by processive ubiquitination. This could reveal if involvement of E4 ligase is a general feature of gene expression that requires rapid responses to internal or external signals. In Chapter 4 the discovery of a new ubiquitin ligase in SA signalling that likely regulates WRKY transcription regulators, opens up new avenues in research on SA-dependent gene expression and could reveal potential new targets to improve disease resistance of crops.

Appendix

A.3.1. Alignments of *Arabidopsis* U-box proteins

Alignments of *Arabidopsis* U-box proteins indicating *At*UBE4 is homologous to *Sc*UFD2 (Figure1 and Figure2) and it is the only member of its class in *Arabidopsis* (Figure 2). Taken from Azevedo et al (2001).

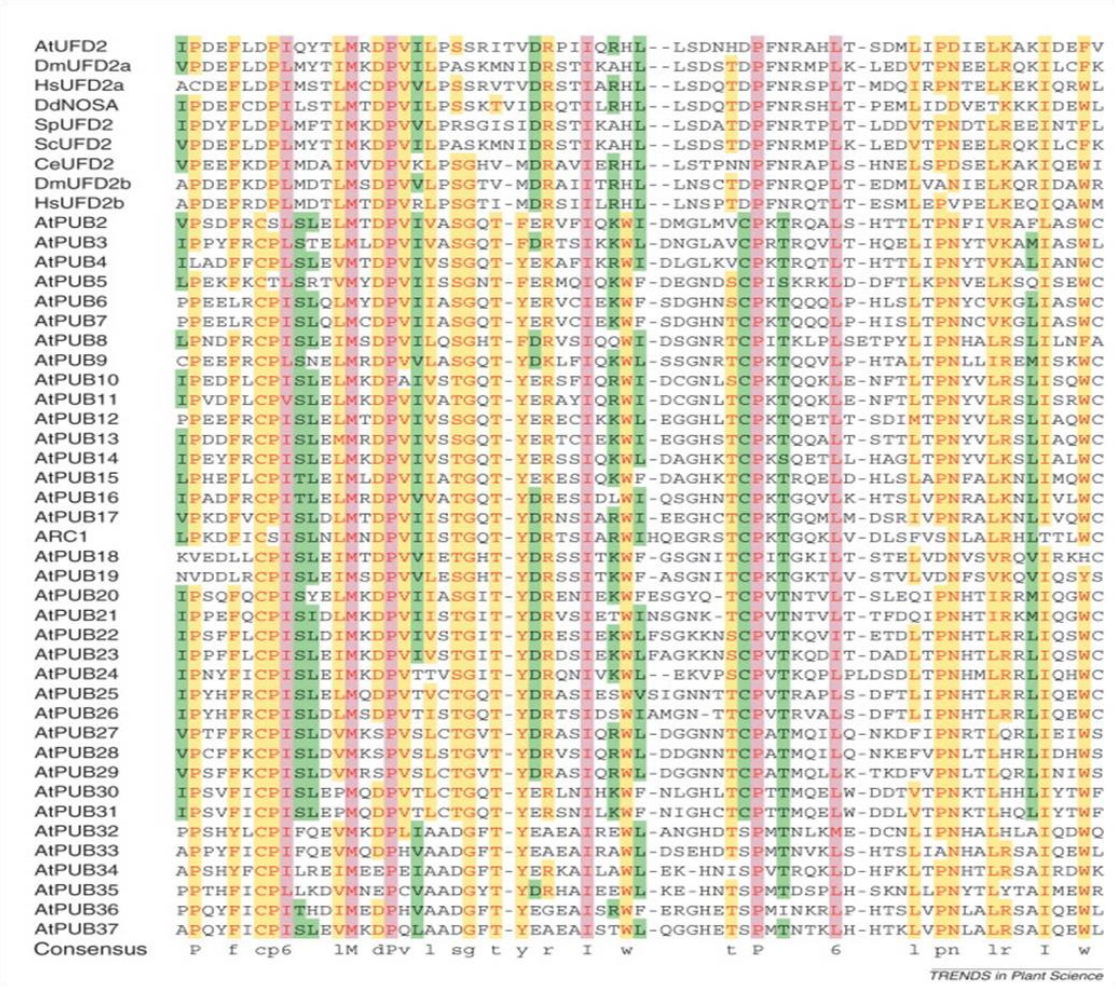

Fig. 1. Alignment of U-box domains. Abbreviations: At, Arabidopsis; Ce, Caenorhabditis elegans; Dd, Dictyostelium discoideum; Dm, Drosophila melanogaster; Hs, human; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe. The alignment was performed using the MultAlin program followed by GeneDoc for coloring . Pink, yellow and green boxes represent 100%, ≥80% and ≥60% functionally conserved amino acids, respectively. The consensus is listed below the alignment. '6' represents either I, L, M or V.

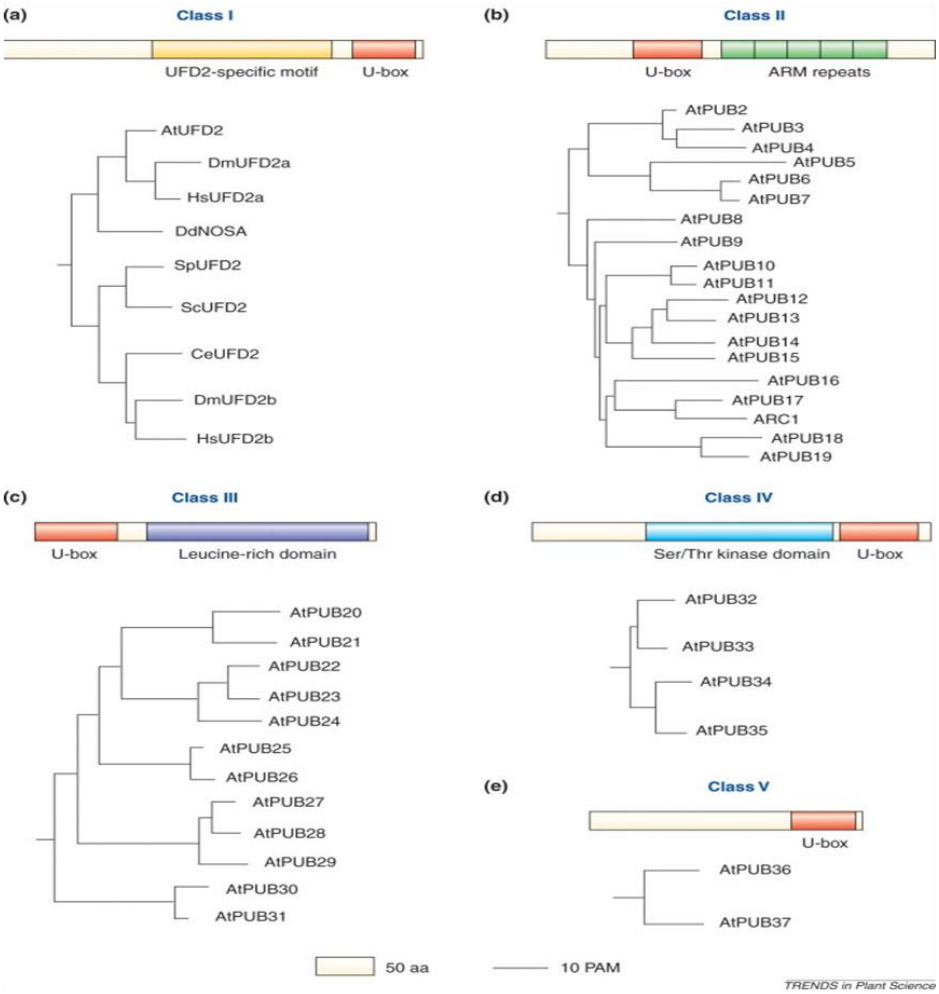

Fig. 2. Domain structures and phylogenetic trees illustrating the relationship of *UFD2* orthologs and the *PUB* gene family in *Arabidopsis*. (a) Class I PUB proteins; *UFD2* orthologs. (b) Class II PUB proteins. (c) Class III PUB proteins. (d) Class IV PUB proteins. (e) Class V PUB proteins. The red, yellow, green, indigo, bright-blue and cream boxes represent the U-box domain, UFD2-specific motif, ARM repeat, leucine-rich domain, Ser/Thr kinase domain and regions with no strong similarity, respectively. The phylogenetic tree was constructed using the MultAlin program [26]. PAM, percent accepted mutations. These were inferred from the types of changes observed in these proteins.

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