An Investigation into the Role of the Ubiquitin-Proteasome System in Plant Defence

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Abbreviations

Amp	Ampicillin Sodium Salt
ATP	Adenosine triphosphate
Avr	Avirulence
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
°C	Degree celcius
Carb	Carbenicillin disodium
cDNA	Complementary deoxyribonucleic acid
dH$_2$O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphate
dsRNA	Double stranded RNA
DTT	Dithiolthreitol
DUB	Deubiquitinating enzymes
EDTA	Ethylene Diamine Tetraacetic Acid
E1	Ubiquitin activating enzymes
E2	Ubiquitin conjugating enzymes
E3	Ubiquitin ligase
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
Fig	Figure
H	Hours
HR	Hypersensitive response
IPTG	Isopropyl-beta-D-Thiogalactopyranoside
Kan	Kanamycin (monosulphate)
Kb	Kilo base
L	Litre
LB	Luria-Bertani
µg	Micro gram
M	Molar
MAMP	Microbial associated molecular patterns
MAP	Mitogen-activated protein
Min	Minute
Mm	Millimetre
Ml	Millilitre
µM	Micro molar

mRNA	Messenger ribonucleid acid
MS	Murashige and Skoog
NaCl	Sodium chloride
PAMPs	Pathogen associated molecular patterns
Pst	*Pseudomonas syringae* pv. *tomato*
PTGS	Post transcriptional gene silencing
PTI	PAMPs triggered imunity
PCR	Polymerase chain reaction
ROI	Reactive oxygen intermediate
RNA	Ribonucleid acid
RNAi	RNA interference
Rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Second
SA	Salicylic acid
SDS	Sodium Dodecil Sulfate
SUMO	Small Ubiquitin like Modifier
TAE	Tris Acetate EDTA pH 8.3
Taq	*Thermus aquaticus*
Tris	Tris (hydroxylmethyl) amino methanes
Tween 20	Polyoxyethylenesorbitan Monolaurate
Ub	Ubiquitin
UBP	Ubiquitin specific potease
UBP_RNAi	UBP12 and UBP13 RNAi
UV	Ultraviolet
Wt	Wild type
X-GAL	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Acknowledgement

Abstract

Ubiquitin is a highly conserved regulatory protein that is ubiquitously expressed in eukaryotes. The ubiquitin pathway appears to be more diversified in plants compared to the other organisms. In plants, the ubiquitin-26S proteasome pathway appears to be particularly involved in regulating plant growth, development and defence signalling.

This project is concerned with establishing potential involvement of UBP12 and UBP13 in plant defence. The *Arabidopsis-Pseudomonas* interaction was employed to examine the transcriptional response of *AtUBP12* and *AtUBP13* during pathogen infection. Furthermore, a transient over expression approach was used to investigate possible gain of function phenotypes associated with *NtUBP12* (tobacco homologue of *AtUBP12* and *AtUBP13*) activity during Cf-9 triggered HR in tobacco. The second objective of this study was to identify the interactor of *UBP12* by employing a yeast two hybrid screen. The final objective was to analyse the functional significance of the mutant forms of ubiquitin during ubiquitination of target protein. Added to the above-mentioned objectives, this study also details the production of plant anti-ubiquitin antibody.

The data presented in this study suggests that *UBP12* and *UBP13* play a critical role in plant defence. The yeast two hybrid assays showed that *DIN1* is the interactor of *UBP12*. The analysis mutant forms of ubiquitin did not show any considerable difference in the pattern of *in vitro* ubiquitination compared to the wild type ubiquitin; the probable reasons for this were discussed. The anti-ubiquitin antibody generated in this study was shown to have affinity against plant and human ubiquitin.

CHAPTER 1

Introduction

1.1 The role of plant defences against pathogenesis

Plants and animals have complex innate mechanisms to recognise and respond to invasion by pathogenic microorganisms (Ausubel, 2005). Plants, unlike animals lack mobile defender cells and a somatic adaptive immune system (Jones and Dangl, 2006). Instead they rely on the innate immunity of individual cells and systemic signals that disseminate from infection sites (Jones and Dangl, 2006) (Liu and Coaker, 2008). Two different branches of the plant innate immune system have been identified (Jones and Dangl, 2006).

One branch of plant innate immune system mediates the perception of microbial or pathogen associated molecular patterns (MAMPs or PAMPs) (Schwessinger and Zipfel., 2008). Defence response activated by PAMPs are collectively termed PAMPs triggerred immunity (PTI) (Schwessinger and Zipfel., 2008). The second branch of the plant innate immune system consists of resistance (R) proteins that specifically recognise the presence of corresponding pathogen effector proteins. This branch of the immune system is termed effector-triggered immunity (ETI) (Liu and Coaker, 2008).

This current view of the plant immune system was described by Jones and Dangl using four phased 'zig zag' model to illustrates relationship between PTI and ETI (figure 1.1) (Jones and Dangl, 2006). During phase 1, early PAMP based perception of pathogen components enables PTI to stop further infection. In phase 2, successful pathogens promote virulence by releasing effectors into the plant cell which suppress PTI resulting in effector-triggered susceptibility (ETS). In phase 3, direct or indirect perception of pathogen effectors by R proteins leads to the activation of ETI resulting disease resistance. Phase 4 depicts ongoing natural selection that drives pathogens to avoid ETI either by discarding or diversifying the recognized effector gene, or by lateral

acquisition of additional ETI supressors. Ultimately, selection favours the generation of new R gene alleles that recognize novel effectors allowing the restoration of ETI (Jones and Dangl, 2006).
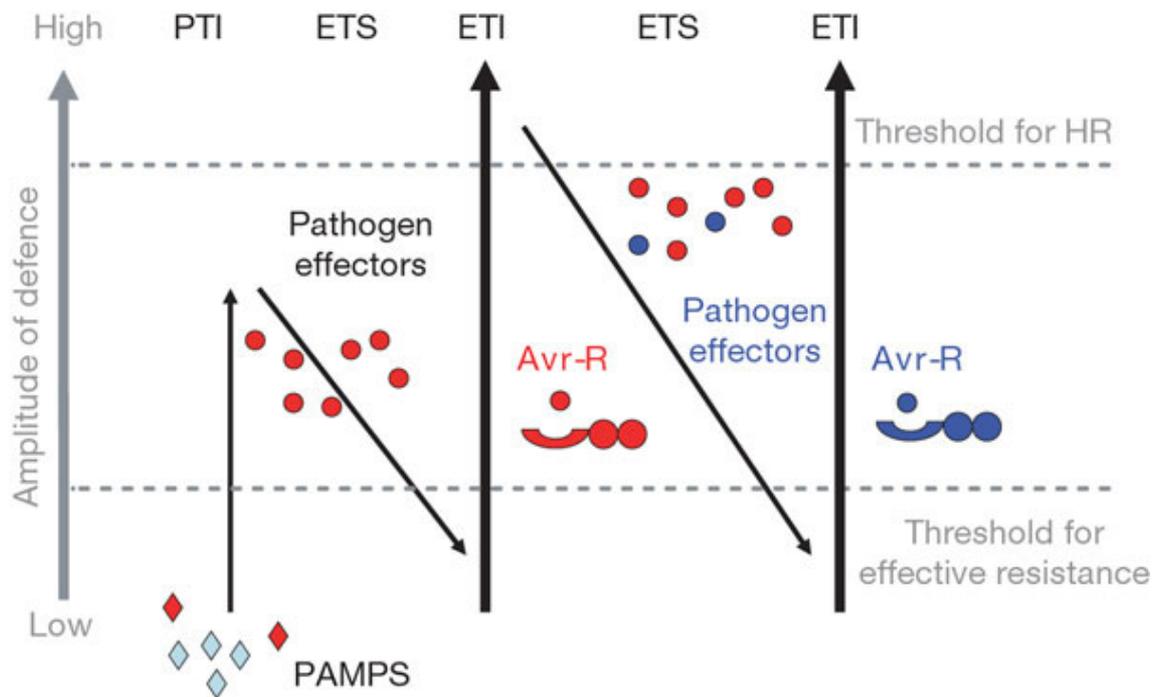
Figure 1.1 A zigzag model illustrates plant immune system output.

In this model, the ultimate amplitude of disease resistance or susceptibility is proportional to [PTI – ETS + ETI]. In phase 1, plants detect microbial/pathogen-associated molecular patterns (MAMPs/ PAMPs, red diamonds) via pattern recognition receptors (PRRs) to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue)—these can help pathogens to suppress ETI. Selection favours new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI.

Adapted from: Jones, J.D.G., and Dangl, J.L. (2006). The plant immune system. Nature, 444, 323-329.

Signal transduction during PTI

Basal resistance (PTI) trigggered by PAMP perception represents the front line of inducible defence responses. These include rapid changes in intracellular Ca^{2+} flux, induction of an oxidative burst, transcriptional reprogramming, cell wall reinforcement and receptor endocytosis (Schwessinger and Zipfel, 2008). PAMP triggered PTI also results in the induction of mitogen-activated protein (MAP) kinase signalling (Liu and Coaker, 2008). Microarray analysis indicates that PAMP perception induces rapid changes in gene expression with a significant expression overlap during PTI induced by fungal or bacterial PAMPs (Zipfel et al., 2006). Significant overlap has also been reported between PTI and ETI transcriptomes underscoring the fact that ETI includes amplified aspects of the PTI response (Zipfel et al., 2006).

Signal transduction during ETI

Gene for gene resistance (as part of ETI) is superimposed onto basal resistance mechanisms and is characterised by a sustained burst of reactive oxygen intermediate (ROI), induction of localised cell death (HR) with activation of defence gene expression and resistance in systemic tissues (systemic acquired resistance) (Jones and Dangl, 2006). Key proteins that regulate ETI have been identified in *Arabidopsis* with isolated mutants indicating that R protein activation leads to activation of the oxidative burst, causing a change in cellular redox status which induces HR and salicylic acid (SA) accumulation (Nimchuk et al., 2003). Elevated SA levels potentiate the HR and lead to the induction of defence genes and the subsequent development of SAR (Nimchuk et al., 2003). Signal transduction events which cause disease resistance following R protein activation during ETI occur through multiple interacting pathways which are regulated by increased transmembrane ion flux (Ca^{2+}, K^+ and H^+), nitric oxide production and increased SA accumulation amongst many other factors (Hofius et al., 2007).

Regulatory feedback and cross-regulation between signalling pathways are recurring themes in plant disease resistance signalling. Current analysis suggest that defence responses are highly regulated in timing and amplitude against specific pathogens or general elicitors by the interaction of many

discrete pathways including hormone signalling, redox control and transcriptional reprogramming (Hofius et al., 2007).

1.2 The ubiquitin 26S proteasome system

1.2.1 Discovery and background

Proteins are the most abundant organic compounds present in all living organisms. After the initial discovery of proteins in the 19th century by Jakob Berzelius it took nearly a century for researchers to determine the three-dimensional structures and analyse their functions. Much work has focused on protein synthesis and little attention was paid to proteolysis (protein degradation) (Hersko, 2004).

For a very long time most intracellular proteins were believed to be long-lived. The dynamic turnover of proteins in the cells, a precise balance of synthesis and degradation, was discovered by Rudolf Schoenheimer in 1942 much earlier than the complete elucidation of the mechanism of protein synthesis. The biochemical mechanisms underlying the process of protein turnover were neglected for a long time (Hersko and Ciechanover, 1992). Experimental evidence accumulated which indicated that intracellular protein degradation is extensive, selective and has basically important cellular functions. Properties of intracellular protein degradation and the role of this process in the regulation of the levels of specific proteins were summarized in a review by Schimke and Doyle in 1970 (Hersko, 2004). In 1978, Hersko and his collegues resolved a heat-stable polypeptide required for the activity of an ATP-dependent proteolytic system from reticulocytes (Hersko and Ciechanover, 1992). This polypeptide was subsequently identified as ubiquitin, a 76-amino-acid, highly conserved protein present in all eukaryotes. The fundamental role of the ubiquitin - 26S proteasome pathway in various cellular processes was first made clear by Varshavsky and co-workers in mammalian cells and yeast (Finley and Varshavsky, 1985).

Following the complete elucidation of the ubiquitin – 26S proteasome pathway much research was carried out to characterise the properties of ubiquitin. The research showed that ubiquitin – 26S proteasome pathway is highly conserved between plants, animals and fungi which indicates the importance of this pathway in normal functioning of the cell. Most of the components of the ubiquitin conjugation pathway and the subunits of the proteasome have been identified in plants (Vierstra, 1996). The ubiquitin pathway which was initially thought to be only involved in protein degradation was later shown to be involved in various non-degradative processes. The ubiquitin – 26S proteasome pathway is particularly important in plants for developmental regulation by selectively removing various cell-cycle effectors, transcription factors, and cell receptors such as phytochrome A (Vierstra, 1996) and recently it has been shown that the ubiquitin – 26S proteasome pathway plays a crucial role in plant defence.

1.2.2 The ubiquitin protein

Ubiquitin (figure 1.2) is a small (8.5 kDa) polypeptide which can be attached to target protein through its carboxy-terminal glycine residue (Sullivan et al., 2003). Only three amino acids differ between mammalian and yeast amino acid residues of ubiquitin (Wilkinson, 2000) (Behuliak et al., 2005) and this indicates the extent of conservation. In plants, ubiquitin plays an important role, and it seems difficult to find biological processes that do not have some connection to ubiquitination (Sullivan et al., 2003).

C-terminus
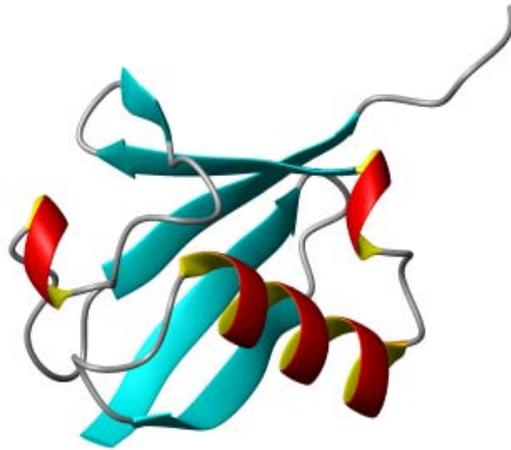
Figure 1.2 The ubiquitin tertiary structure.

A glycine residue is exposed at the C-terminus end and will ultimately form an isopeptide bond with target proteins.

Adapted from: Kumar et al. (1987). Structure of ubiquitin refined at 1.8 Å esolution. J. Mol. Biol., 194 (3) 531-544.

Ubiquitin contains seven lysines (K6, K11, K27, K29, K31, K48 and K63). To target substrates for degradation by the proteasome, covalent inter-ubiquitin linkages are made from the C-terminal glycine to the K48 of the previous ubiquitin moiety (i.e. G76-K48 isopeptide bond) to form ubiquitin chains (poly-Ub) (Fushman and Pickart, 2004). Poly-Ub chains of at least four ubiquitin moieties (tetra-ubiquitin) are required to provide an efficient proteasome delivery signal (Thrower et al., 2000).

1.2.3 The ubiquitin conjugation cascade

The two discrete steps involved in the ubiquitination pathway are tagging of the substrate with polyubiquitin molecules and degrading the target protein in the 26S proteasome complex. The components of the ubiquitin pathway are classified into: ubiquitin activating enzyme E1, ubiquitin carrier or conjugating enzyme E2 and ubiquitin-protein ligase E3. The following schematic diagram (figure 1.3) briefly describes the role of E1, E2, E3 in the ubiquitin pathway. The cascade starts with an E1 enzyme. The E1 enzyme activates ubiquitin by utilizing energy derived from ATP hydrolysis and forms an ubiquitin-adenylate intermediate followed by transfer of ubiquitin to a cysteine residue, with release of AMP. (Hersko and Ciechanover, 1982). A thiolester linkage is formed between the C-terminal carboxyl group of ubiquitin and the E1 cysteine sulfhydryl group. The activated ubiquitin is subsequently transferred to the cysteine residue on a ubiquitin conjugating enzyme (E2) by transesterification. The E2-ubiquitin intermediate then delivers the ubiquitin onto a substrate acceptor lysine, usually using an E3 (Ub-protein ligase) as the catalyst (Hersko and Ciechanover, 1992). The end product is an Ub-protein conjugate containing an isopeptide bond between the C-terminal glycine of Ub and lysyl ϵ-amino group in the substrate. By successively adding activated ubiquitin moieties to internal Lys residues on the previously conjugated ubiquitin molecule, a polyubiquitin (poly-Ub) chain is synthesized (Glickman and Ciechanover, 2002). It is currently unclear, whether ubiquitin chains are extended by ligation of pre-assembled poly-Ub or by iterative rounds of E3 based ligation (Smalle and Vierstra, 2004).

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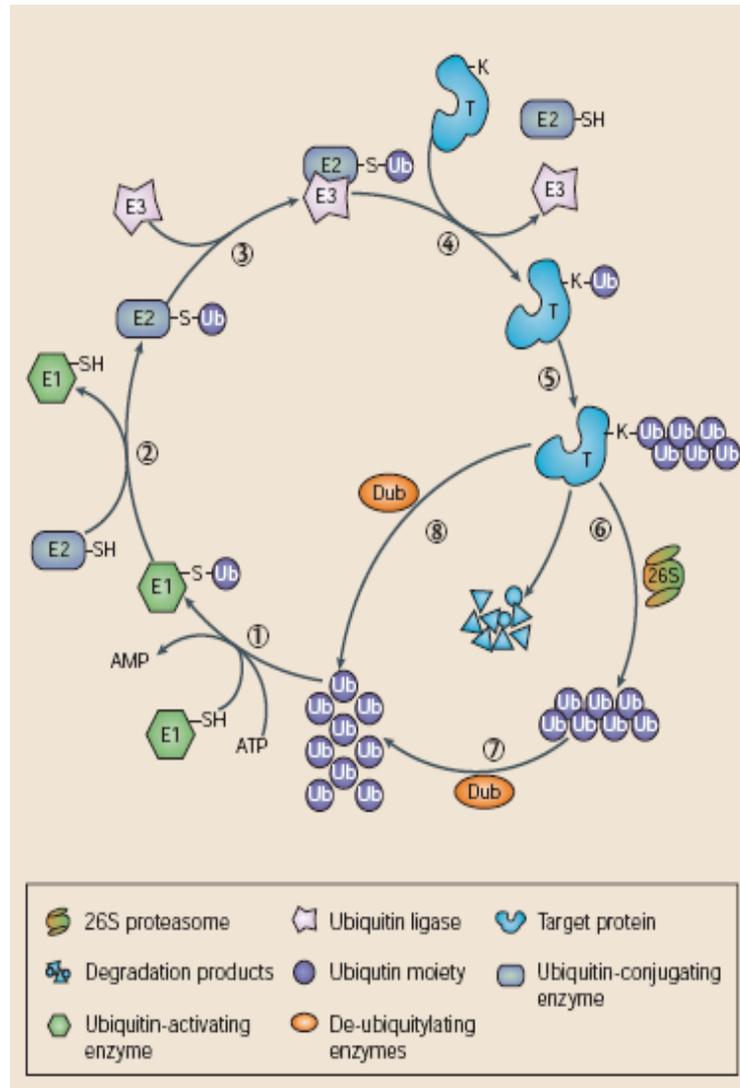
Figure 1.3 Ubiquitin conjugation cascade.

(1) ATP dependent activation of Ub by E1. Activated Ub binds to a conserved cysteine in E1 via a thiolester linkage from a carboxy group in its terminal glycine. (2) Transfer of Ub to Ubiquitin conjugating enzyme (E2) forming an E2-Ub thiolester linkage. (3) The E2 carries the activated ubiquitin to the ubiquitin ligase (E3),which facilitates the transfer of the ubiquitin from the E2 to a lysine residue in the target protein, often by forming an intermediate complex with the E3 and the target. (4) Initial ubiquitination of target forming a Ub-protein conjugate linked by a isopeptide bond. (5) Additional Ubs are ligated to form poly-Ub chains. Proteins tagged with K48 linked poly-Ub chains are targeted to the proteasome. (6, 7) Poly-Ub chains are disassembled by a proteasome associated deubiquitinating activity (DUB) and free Ub moieties are released. (8) Proteasome localised substrates are then unfolded, imported and degraded into peptide fragments

Adapted from: Sullivan et al. (2003). The diverse roles of ubiquitin and the 26S proteasome in the life of plants. Nature, 4, 948-958.

E1 Ubiquitin activating enzyme

The E1 enzyme initiates the Ub conjugation cascade but has a negligible effect on its regulation. The E1 enzyme is a single polypeptide of ~1100 residues that contains a conserved cysteine which binds the activated Ub (Hatfield et al., 1997). The *Arabidopsis* genome encodes two ubiquitin activating enzymes E1 enzymes AtUBA1 and AtUBA2 (Hatfield et al., 1997).

E2 Ubiquitin conjugating enzyme

The ubiquitin-conjugating enzyme, E2 accepts the ubiquitin protein from the E1 enzyme and transfers it to the target protein or to the E3 enzyme (Glickman and Ciechanover, 2002). Multiple E2 enzymes are reported in eukaryotic organisms. The E2 enzymes share an ubiquitin binding active cysteine residue and share a conserved region of 140-150 amino acid region called the ubiquitin conjugating (UBC) domain (Kraft et al., 2005). The E2 enzymes are shown to interact with the substrate directly in some cases but the physiological significance of the interaction is unknown (Kalchman et al., 1996). Most of the E2 enzymes are broadly expressed in *Arabidopsis* with each enzyme having a unique spatial and developmental pattern of expression (Kraft et al., 2005).

The functions of E2 enzymes in plants are not well characterized. The Arabidopsis genome encodes for 37 E2 enzymes of which only 9 enzymes are known to form an ubiquitin thiolester bond with ubiquitin (Bachmair et al., 2001). Little is known about the ability of the E2 enzymes to bind with various E3 enzymes, their modes of interaction, and their significance in the plant life cycle. The E2 enzymes are assumed to be capable of interacting directly with protein to be ubiquitinated or the E3 enzymes. Several E2 enzymes are reported to have the ability to interact with multiple E3 enzymes (Ptack et al., 2001).

E3 Ubiquitin ligase

The ubiquitin ligase, E3 is a protein or a protein complex that binds to both the E2 and the substrate (Glickman and Ciechanover, 2002). E3s are the key players in exhibiting the high specificity and selectivity of ubiquitination. In order to confer substrate selectivity for an extensive range of substrates, the E3s are the most diverse proteins in the ubiquitination cascade (Varshavsky, 1996). The *Arabidopsis* genome contains over 1300 genes that encode putative E3 subunits with one family containing almost 700 members (Vierstra, 2003). To date, there are currently five types of known ubiquitin ligases: VBC-Cul2, HECT, Ring/U-box, SCF and APC, the latter four of which have been identified in the *Arabidopsis* genome (Smalle and Vierstra, 2004).

Homology to E6AP C-Terminus (HECT) E3s are single polypeptides that were originally identified by the presence of a conserved 350-amino acid C-terminal HECT domain (Vierstra, 2003). This domain contains a conserved Cys residue to bind specific E2s, accepts ubiquitin from the E2 to form a ubiquitin-thiolester intermediate with the HECT active cysteine, and then transfers ubiquitin to either the amino group of lysine side chains of the substrate or to the growing end of multiubiquitin chains (Xie and Varshavsky, 1999). Genome analysis has identified 7 HECT E3s in *Arabidopsis,* 5 in yeast and more than 50 in human (Downes et al., 2003).

Really Interesting New Gene (RING) domain interacts with E2-Ub intermediates using variants of a zinc-finger structure (Kosarev et al., 2002). The RING motif is formed by an octet cysteines and histidines that binds zinc in either a RING-H2 or ring HC arrangement. U-box proteins have a predicted three-dimensional structure similar to the RING-finger but lack the specific zinc-chelating residues (Ohi et al., 2003). The U-box domain was first identified at the C-terminus of the yeast UFD2. Numerous RING/U-box encoding genes have been detected in the Arabidopsis genome with around 500 RING and 130 U-box proteins currently identified (Mudgil et al., 2004)

Skp1-Cul1-F-box protein (SCF) E3s are involved in targeting protein substrates for phosphorylation and were originally found in *Saccharomyces cerevisiae* (Deshaies 1999) (Glickman and Ciechanover, 2002). SCF E3s are made up of

four polypeptides: SKP1, CDC53 (Cullin), F-box protein and RBX1 (or ROC1 and HRT1) (Deshaies, 1999). The F-box motif facilitates interaction between substrates and ubiquitin-conjugating enzymes, which then covalently transfer ubiquitin onto substrates (Kipreos and Pagano, 2000). SCF complexes appear to be constitutively active E3 enzymes that recognise and ubiquitinate only phosphorylated substrates (Vierstra, 2003). SCF E3s are implicated in numerous plant signalling pathways including responses to the plant hormones, cell cycle progression and photomorphogenesis (Schwechheimer and Villalobos, 2004). Using published F-boxes as queries 694 potential F-box genes in *Arabidopsis* have been identified, making this gene superfamily one of the largest currently known in plants (Gagne et al., 2002).

Anaphase Promoting Complex (APC) is the most elaborate E3 family, first identified in yeast and metazoans by its role in the timed breakdown of cyclins and several mitotic checkpoint proteins (Vierstra, 2003). Subsequently, the role of APC in degrading other crucial cell cycle regulators was discovered (Capron et al., 2003) and the name cyclosome was assigned to the complex. *Arabidopsis* orthologs have been detected for most APC subunits (Capron et al., 2003) which are predominantly present in single copies suggesting that a limited number of APC isoforms are assembled.

1.2.4 The 26S proteasome

The 26S proteasome is a 2 MDa ATP dependent proteolysis complex which degrades ubiquitin tagged substrates. Whilst initial characterisation of the complex was derived from studies of yeast and mammalian proteasomes, subsequent studies in rice and *Arabidopsis* indicate a similar design (Hu et al., 1998).

The 26S proteasome can be further divided into two particles, the 20S core protease (CP) and the 19S regulatory particle (RP) (Vierstra, 2003). The proteolytic activity of the proteasome is found within the 20S CP (Glickman and Ciechanover, 2002). Although purified free 20S CP can hydrolyze small peptides and some unfolded proteins, it cannot degraded multi-ubiquitinated proteins. The structure of the 20S CP purified from yeast was determined by X-ray crystallography. The CP is a hollow cylindrical structure composed of

four heptagonal rings stacked in C_2 symmetry (Groll et al., 1998). Each end of the CP is capped by a RP; the RP confers both ATP-dependence and substrate specificity to the holloenzyme, especially with respect to those bearing the poly-Ub tag (Vierstra, 2003). The RP serves multiple roles in regulating proteasomal activity: selecting substrates, preparing them for degradation, translocating them into CP, as well as probably influencing the nature of product generated by the CP (Glickman and Ciechanover, 2002).

1.2.5 Deubiquitination enzymes

Protein ubiquitination is a reversible process, and it has become increasingly obvious that Ub deconjugation plays important roles in regulating the Ub-dependent pathways (Chung and Baek., 1999) (Wilkinson., 1997). Deubiquitinating enzymes (DUBs) catalyze the removal of Ub from Ub-conjugated substrate proteins. DUBs (multiple deubiquitylation enzymes; isopeptidases) are thiol proteases that remove Ub enabling the Ub used in labelling proteins to be recycled and reused in the Ubiquitin-26 Protease pathway. Plants, animals and yeast contain a family of DUBs capable of specifically removing covalently bound Ubs. These DUBs help regulate the Ub-26S proteasome pathway by generating free Ub moieties from their initial translation products, recycling Ub during breakdown of the Ub-protein conjugates, and or removing Ubs from specific targets and thus preventing their turnover by the 26S proteasome (Vierstra, 2003). DUB enzymes perform several important functions in the Ub–26S proteasome pathway (figure 1.4).

Deubiquitination negatively regulates protein degradation by hydrolyzing K48-linkages and disassembling the poly-Ub chains from the target proteins. It serves as a proofreading mechanism rescuing proteins inappropriately targeted for degradation (Americk and Hochstrasser, 2004). DUB enzymes also process Ub precursors via cleavage of polyUb fusions or Ub fusions to unrelated proteins (Callis et al., 1990) (Americk and Hochstrasser, 2004). Moreover, they prevent free Ub molecules from being attacked by small intracellular nucleophiles such as glutathione and polyamines (Americh and Hochstrasser, 2004).
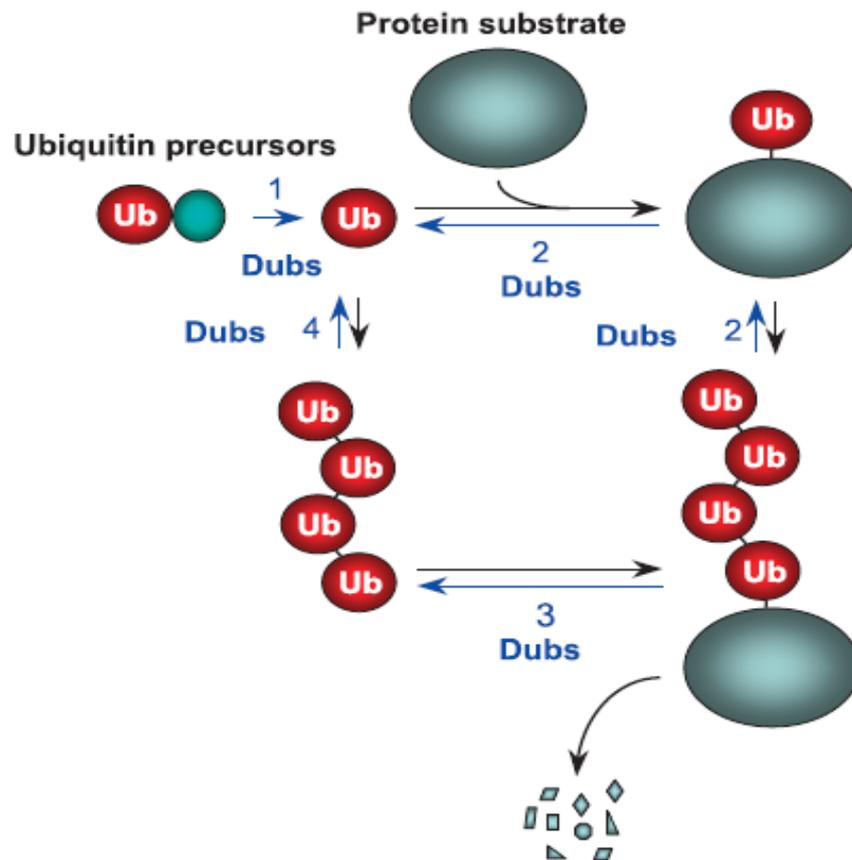
Figure 1.4 Functions of DUBs in the ubiquitin pathway.

(1) Processing of ubiquitin precursors. (2) Editing or rescue of ubiquitin conjugates, which are generally adducts to other proteins in the cell but can also be ligated to abundant small nucleophiles such as gluthathione. (3) Recycling of ubiquitin or ubiquitin oligomers from ubiquitin-protein conjugates targeted for degradation. (4) Disassembly of unanchored ubiquitin oligomers.

Adapted from: Amerik and Hocstrasser. (2004). Mechanism and function of deubiquitinating enzymes. Biochemica et Biophysica Acta 1965, 189-207.

DUB enzymes are classified into at least five subfamilies based on sequence homology and catalytic mechanism. Four of the subfamilies represent specialized cysteine proteases with a 'papain type' fold. They include ubiquitin carboxyl hydrolases (UCHs), ubiquitin specific processing proteases (UBPs), machado-joseph disease protein domain proteases (MJDs), ovarian tumor proteases (OTU) (Nijman et al., 2005) and the fifth subfamily is JAB1/MPN/Mov34 metalloenzyme (JAMM) motif proteases (Americk and Hochstrasser, 2004) (Nijman et al., 2005).

Ubiquitin Carboxyl Hydrolyses (UCHs)

UCHs were the first described DUBs, their specific function remain poorly understood (Nijman et al., 2005). UCH are thought to mainly act in the recycling of Ub when Ub is inappropriately conjugated to intracellular nucleophiles. Together with other DUBs they may be involved in the processing of newly synthesized Ub, which is translated either as a polyubiquitin precursor or fused to ribosomal protein precursors (Nijman et al., 2005).

Ubiquitin Specific Proteases (UBPs)

UBPs are a family of unique hydrolases that specifically remove polypeptides covalently linked via peptide or isopeptides bonds to the C terminal glycine of ubiquitin (Yan et al., 2000). This family is the largest and most diverse of DUB enzymmes (Amerik and Hochstrasser, 2004). Over 35 UBP encoding genes have been identified in *Arabidopsis thaliana* (Ewan., 2008).

Machado-Joseph Disease Protein Domain Proteases (MJDs)

Experiment *in vitro* confirmed that wild type Ataxin-3 could deubiquitinate a model substrate. Even though sequence similarity between the catalytic domain of Ataxin3 and other DUBs is low, NMR structures show that the overall arrangement of the catalytic triad is conserved (Nijman et al., 2005).

Ovarian Tumor Proteases (OTUs)

A bioinformatics approach led to the identification of Ovarian Tumor (OTU), an additional subclass of DUB enzyme (Makarova et al., 2000). Five OTU types have been detected in human, three have experimentally confirmed DUB activity (Balakirav et al., 2003). Otubain 1 and Otubain 2 were the first two OTU proteins found to display in vitro DUB activity (Nijman et al., 2005).

JAMM Motif Proteases

The JAMM domain is found in all three major kingdoms of life (bacteria, archaea, and eukarya) (Nijman et al., 2005), it represents a recently identified class of novel zinc metalloprotease DUBs (Amerik and Hochstrasser, 2004). JAMM domain subunits have been identified as key components of the proteasome regulatory particle (RPN11) (Verma et al., 2002), COP9 signalosome (CSN5) (Cope et al., 2002), eIF3 translation initiation complex (Glickman et al., 1998) and STAM endocytotic regulatory complex (McCullough et al., 2004). In the above cases JAMM domain proteins have been proven to facilitate hydrolysis of Ub or Ubiquitin-like moieties (eg RUB-1) with RPN11 proving to be essential for viability in yeast (Verma et al., 2002). Homologs of all currently known JAMM DUBs have been detected in *Arabidopsis* (Ewan., 2008).

1.3 Ubiquitination in plant defence signalling

Ubiquitin mediated proteolysis is known to be a central regulatory mechanism in the control of several cellular processes in many eukaryotes (Devoto et al., 2003). Ubiquitination has also been implicated in a growing number of plant signalling pathways linked to hormone signalling, growth, development and also plant defence signalling (Vierstra, 2003) (Devoto et al., 2003).

Involvement of E3 ubiquitin ligases in plant defence

Ubiquitin ligases account for the specificity of the ubiquitin pathway (Gray, 2002). Several genes encoding RING type and F-box protein, E3 ubiquitin ligases have been shown to be active participants in defence signalling pathways. Two F-box proteins, coronatine intensive 1 (coi1) and supressor of nim1-1 (son1) are shown to be essential regulators in plant defence. Coi1 controls defence pathways that are regulated by jasmonic acid (JA), which is a signalling molecule that coordinates plant responses to numerous biotic and abiotic stresses. Son1 regulates a novel induced defence response that is independent of both salicilic acid and SAR (Xie et al., 1998) (Kim and Delaney, 2002).

Numerous RING-type E3 ubiquitin ligase have been identified as candidates for involvement in defence signalling pathways. For example, the *ATL2* and *ATL6* genes encode putative RING-finger proteins that are induced rapidly in *Arabidopsis* after elicitor treatment (Devoto et al., 2003).

Two U-boxes E3 ligases were identified in the A̲vr9/C̲f-9 R̲apidly E̲licited (ACRE) screen. The two ligases identified ACRE276 and ACRE74, act as positive regulators of ETI (Yang et al., 2006) (Gonzales-Lamothe et al., 2006). ACRE276 found in tobacco and tomato, appears to be required for Cf-9 stimulated hypersensitive response (HR) because after virus-induced gene silencing of *ACRE276,* tomatoes have increased susceptibility to *Cladisporium vulvum* leaf mould (Yang et al., 2006). The closest homologue of tobacco ACRE276 in plants is *Arabidopsis* PUB17 (70% identity over the entire protein sequence) (Yang et al., 2006). PUB17 was also implicated in plant defence with *pub17* mutants demonstrating increased susceptibility to avirulent strains of *Pseudomonas syringae* (Yang et al., 2006). Similar experimental approaches have also demonstrated that ACRE74 mediates Cf-9 triggered HR and resistance (Gonzales-Lamothe et al., 2006). *ACRE74* encodes a U-box E3 ligase homolog, highly related to parsley (*Petroselinum crispum*) CMPG1 and *Arabidopsis thaliana* PUB 20 and 21, and was named NtCMPG1 (Gonzalez-Lamothe et al., 2006). CMPG1 appears to function as a positive regulator of the HR, as tomato and tobacco plants with reduced levels of CMPG1 display reduced HR (Gonzalez-Lamothe et al., 2006).

Role of ubiquitinated substrates in plant defence

There are a few documented cases of plant defense proteins undergoing regulated proteolysis during pathogenic interactions. A potential link has been established in the case of *Arabidopsis* R protein RPM1. RPM1 specifically helps plants to defend themselves against *Pseudomonas syringae* harbouring the *avrRpm1* and *AvrB* genes (Dreher and Callis, 2007). RPM1 is a perispheral plasma membrane protein, and it is rapidly down regulated via an unknown mechanism coinciding with the onset of HR (Kawasaki et al., 2005). Two proteins, RIN2 and RIN3, each with a RING domain and a ubiquitin binding domain called CUE, were shown to interact with RPM1. GST-RIN2 and RIN3 fusion proteins posses E3 ubiquitin ligase activity *in vitro.* Analysis of the *rin2*

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and *rin3* double mutants indicate that these two proteins contribute to pathogen-elicited RPM1-dependent ion leakage (Kawasaki et al., 2005). RIN2 and RIN3 function additively as positive regulators of RPM1 mediated HR (Kawasaki et al., 2005).

Although very few examples of ubiquitinated host proteins have been implicated in plant defence pathways to date, a number of experiments suggest that viral proteins may act as substrates for the host Ubiquitin-26S proteasome system (Dreher and Callis, 2007). A general linkage between ubiquitin and viral/host interactions was revealed in tobacco plants expressing a mutant variant of ubiquitin with lysine 48 changed to arginine. These mutant plants exhibit lesion development and display several alterations in Tobacco mossic virus (TMV) susceptibility and symptom progression (Dreher and Callis, 2007).

1.4 Study objectives

As there is accumulating evidence which implicates the role of ubiquitination in plant defence and disease signalling, the primary aim of this study is to establish the potential involvement of UBP12 and UBP13 in plant defence. The role of UBP12 in *Nicotiana tabaccum* defence signalling was studied by performing transient assays.

Another objective of this study is to identify the interactor of UBP12 (DIN1) using the yeast two hybrid systems. Examine the role of DIN1 using loss of function assay and also generate DIN1-GFP fusion construct to study the subcellular localization of this protein.

The last objective is to develop lysine to arginine mutants of ubiquitin to be used to analyse the function significance of the 7 different lysine residues during ubiquitination of target protein. Finally this study also details the generation of anti-plant Ubiquitin antibody.

CHAPTER 2

Materials and Methods

The following section lists and describes material and methods used in this study.

2.1 Materials

2.1.1 Plant

Arabidopsis seed stocks.
Wild-type *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) seeds were obtained from The National Arabidopsis Stock Centre (NASC, Nottingham, UK). T-DNA insertional mutant lines in the Col-0 genetic background were obtained from NASC (Nottingham, UK) and GABI-Kat (Cologne, Germany).

2.1.2 Enzymes and reagents

All chemicals were used in this study provided by Sigma-Alrich Ltd (Poole, UK), Fisher Scientific UK (Southampton, UK) or VWR International Ltd. (Poole, UK) unless otherwise stated described below. Complete protease inhibitor cocktail Tablets (EDTA-free, cat# 11836170001) were supplied by Roche. Ponceau S powder (cat# P3504), Coomassie Brilliant Blue stain (cat# B8647), His-Select affinity purification columns (cat# H7787), Triton X-100 (cat# T8787), Tween20 (cat# P5927), Murashige and Skoog Basal Medium (cat# M5519) and Rifampicin (cat# R3501) were provided by Sigma-Aldrich. QIAprep plasmid miniprep kit (cat# 27104) and QIAquick gel extraction kit (cat# 28704) were provided by Qiagen Ltd. (Crawley, UK). 37.5:1 acrylamide:bis-acrylamide solution (cat# 161-0156) and Polyvinylidene flouride PVDF membrane (cat# 162-0177) were provided by BioRad Laboratories. Chemiluminescent HRP substrate (cat# WBKLS0500) was provided by Millipore Ltd. TRIzol RNA extraction (cat# 15596026), RnaseOut inhibitor (cat# 10777019) were provided by Invitrogen Ltd. (Paisley, UK). dNTPs (cat# U1330),

RNasin Ribonuclease Inhibitor (cat# N2111) were provided by Promega (Southampton, UK). DNA-free DNAse (cat# 1906) and Nuclease-free H_20 (cat# 9930) were provided by Ambion. Brilliant SYBR Green (cat# 600548) was provided by Stratagene Ltd. Proteose Peptone salts (cat# LP0085) was provided by Oxoid Ltd. Cell culture flasks (cat 43072) provided by Corning (NY, USA). His-Bind affinity purification resin (cat# 69670), BugBuster cell lysis reagent (cat# 70584) were provided by Novagen (Nottingham, UK). Human recombinant E1 (cat#UW9410), ubiquitin conjugating enzymes (cat# UW9050), ATP (cat#UW9805), ubiquitinylation buffer (cat#UW9885), non reducing gel loading buffer (cat#UW9880) supplied by Biomol international (UK). Inorganic pyrophospatase solution (cat#UW83205) was supplied by Fluka. Marker of DNA and protein used in this study are described in table 2.1 below

Table 2.1 Markers

Name	Supplier
1 kB DNA marker	G571A 24302 (Promega)
6.5-175 kDa Protein marker	P 7708 S (Biolabs)

The following table (table 2.2) describes endonucleases that were used in this study.

Table 2.2 Endonuclease enzymes

Name	Restriction site	Supplier
*Asp*718	GGTACC	cat#1175050001 (Roche)
*Bgl*II	AGATCT	cat#11175068001 (Roche)
*EcoR*I	GAATTC	cat#10703737001 (Roche)
*Not*I	GCGGCCGC	cat# 11014706001 (Roche)
*Pvu*II	CAGCTG	cat#R6331 (Promega)
*Sal*I	GTCGAC	Cat#10567663001 (Roche)
*Xho*I	CTCGAG	Cat#10899194001 (Roche)

* The sequence shown is that of one strand, given in the 5' to 3' direction.

Taq DNA polymerase was used for standard PCR. To achieve high accuracy, fidelity and blunt end product, Phusion polymerases were used when PCR

products were generated for cloning. Modifying enzymes and their sources were listed in the table 2.3 below:

Table 2.3 Nucleic acid modifying enzymes

Name	Description
Taq DNA polymerase	cat# M0267S (NEB)
HiFi DNA polymerase	cat# 11732641001 (Roche)
Phusion DNA polymerase	cat# F530S (NEB)
SuperScript^{TM} II RNase H - Reverse Transcriptase	cat# 18064022 (Invitrogen)
Gateway LR Clonase Enzyme mix	cat# 11791100 (Invitrogen)
T4 DNA ligases	cat# M1801 (Promega)

2.1.3 Bacterial strains

Table 2.4 Bacterial strains

Bacterial strains	Description
Escherichia coli DH5α	Sub-cloning, amplification
Escherichia coli BL21 (DE3)	Protein expression
Agrobacterium tumefaciens GV3101	Transient expression
Pseudomonas syringae pv. *Tomato* DC3000	Pathogen assay
Pseudomonas syringae avrRpt2	Pathogen assay

2.1.4 Plasmid vectors

Table 2.5 Plasmid vectors

Plasmid vector	Description	Source
pGEM®-T Easy vector	Sequencing & Sub-cloning	cat# A1360 (Promega)
pENTR^{TM}/D-TOPO	Gateway entry cloning	cat# K240020 (Invitrogen)
pENTR^{TM}4 vector	Gateway entry cloning	cat#11818-010 (Invitrogen)
pET-30a	Histidine tag	cat# 69909-3 (Novagen)
pGWB6	Gateway GFP tag	cat#K410-01 (Invitrogen)

2.1.5 Antibiotics

Table 2.6 Antibiotics were used for plasmid, bacterial and seed selection

Antibiotic	Solvent	Stock Concentration	Working Concentration
Kanamycin	H_2O	50 mg/ml	50 µg/ml
Rifampicin	Methanol	10 mg/ml	100 µg/ml
Carbenicillin	H_2O	100 mg ml	100 µg/ml
Hygromycin B	PBS	50 mg/ml	50 µg/ml

2.1.6 Antibodies

Table 2.7 Antibodies used for western blots in this study

Antibody	Dilution	Source	Incubation	Supplier
Anti-Ubiquitin	1:10000	Rabbit	120 min	cat# 08EF0016 (Agrisera)
Anti-Histidine	1:2000	Rabbit	120 min	cat# SC803 (Santa cruz)
Anti-Rabbit HRP	1:10000	Goat	45 min	cat# A6154 (Sigma)

2.1.7 Oligonucleotides

All primers used in this research were synthesised by MWG and supplied as 100 μM stocks.

Table 2.8 Oligonucleotides

Primer name and specificity	Sequences	Restriction site	Annealing temperature
ACT2a (*At*ACT2,5' end)	CTT ACA ATT TCC CGC TCT GC		55°C
ACT2s (*At*ACT2,3' end)	GTT GGG ATG AAC CAG AAG GA		55°C
dT	TTT TTT TTT TTT TTT TT		41.65°C
Infub30P1	GAT CTA GAA TTC ATG CAG CTC TTT GTG GAG ACC CTA TA	EcoRI	70.5°C
Infub30P2	GGT CTA CTC GAG TCA ACC ACC GTT CAC AGA CAG CAG CAT C	XhoI	74.6°C
lba1	TGG TTC ACG TAG TGG GCC ATC G		75°C
Nt UBP12.3	CGC CAC TCT CTC CAC TAC ATC		56°C
Nt UBP12.5	GAA TTG GCT GGA TTT GCT CCT G		55°C
RTP1	CACC ATG GCG TGC TCA ATT GGT AAC GCT		68°C
RTP2	CTT CAC CCA ATG TGT TTC TGT CCC		62.7°C
RTP3	TTC TGA TAC GCT GCG TTT TG		55.3°C
RTP4	AAA CTG AGG ATG CGT GTT CC		57.3°C
UBP12C207S.3	CTG TAG GAG AGA ATT CAT GTA GCT TGT TGC ACC TTG		64°C
UBP12GW.5	CAC CAT GAC TAT GAT GAC TCC GCC TCC CGT T		66°C
UBP13.3	TCC TTC TGC ATG GTA TTT GTT G		51°C
UBP13.5	GAT GCA CCT ACC GCT AGT ATC C		57°C

- The sequence shown is given in the 5' to 3' direction.

2.1.8 General laboratory solution

All the buffers and solutions composition were used in this study were shown in the list below:

Coomassie Stain
1gR-250
250ml Methanol
37.5 ml acetic acid
H_2O to 500 ml

Coomassie Destain
500ml H_2O
400ml Ethanol
100ml acetic acid

DNA extract. buffer
pH 7.5
200 mM Tris
250 mM NaCl
25 mM EDTA
0.5% SDS

DNA loading dye (6x)
4g Sucrose
2 ml EDTA (0.5 M)
25 mg Bromophenol blue
H_2O to 10 ml

Ponceau S
0.1% in 1% acetic acid

Resolving SDS-PAGE
5 ml1.5 M Tris
×ml 30% acrlyamide
0.2 ml 10%SDS
0.1 ml APS
10 µl temed
×ml H_2O

Running buffer(10×)
30.5 g Tris
139 g Glycine
10g SDS
H_2O to 1000 ml

Running buffer (1×)
100 ml 10×running buffer
H_2O to 1000 ml

TBS pH 7.6 (10×)
Tris 20 mM
NaCl 150 mM

TBST (1×)
100 ml 10×TBS buffer
1 ml Tween20
H_2O to 1000 ml

Stacking SDS-PAGE
2.5ml 0.5 M Tris
1.33ml 30% acryamide
100 µl 10%SDS
100 µl APS
10 µl temed
6ml H_2O

SDS buffer (4×)
2ml 2-mercaptoethanol
0.8g SDS
2ml 0.5M tris
0.4% pinch Bromphenol blue
4ml glycerol
Make up to 10ml H_2O

Transfer buffer(10×)
30.5 g Tris
139 g Glycine
H_2O to 1000 ml

Transfer buffer (1×)
100 ml 10×transfer
200 ml methanol
H_2O to 1000 ml

All the media were used in this study were shown in the list below:

1/2 MS media pH 5.8
2.2g MS(#Sigma M5519)
7.5g sucrose
Make up to 1L H_2O
Add agar 1%

LB Media pH 7
10g tryptone
10g NaCl
5g yeast extract
Make up to 1L H_2O
For solid LB add agar 15g/L

Kings media pH 7.2
40g peptone
24ml glycerol
3g K_2HPO_4
3g $MgSO_4.7H_2O$
Make up to 2L H_2O
Add agar 15g/L

2.2 General laboratory procedures

2.2.1 Autoclaving

Solutions and equipment were sterilised in benchtop (eg. Prestige Medical, Model 220140) or free-standing (eg. Laboratory Thermal Equipment Autoclave 225E) autoclaves.

2.2.2 pH Measurement

The pH of solutions and media were measured using a Metler Toledo MP220 pH meter and glass electrode.

2.3 Plant growth media and conditions

2.3.1 Sterilisation of *Arabidopsis thaliana* seeds

Arabidopsis thaliana seeds were sterilized under a fume hood overnight. Desired amounts of seeds were placed in eppendorf tubes labelled with pencil (because marker ink can sometimes fade). 100 ml of bleach solution was prepared in a small beaker then placed in desiccators. Racks of tubes containing seeds with lids open were added. To avoid cross contamination, the tubes in the rack were spaced out. 3ml of concentrated HCl was added to the beaker containing bleach solution and quickly replaced the lid (while chlorine gas is being generated) on desiccators.

2.3.2 Growing *Arabidopsis thaliana* seeds on agar plates

On the following day, the seeds were taken from the fume hood; they were added to 1 ml sterilized dH_2O and sprayed onto agar plates containing the desired media with appropriate antibiotics. The plates were wrapped with parafilm and were stored in the refrigerator with the agar side up (seeds do not fall). The plates were also wrapped with aluminium foil to ensure complete darkness to allow induction of germination. After 3 days incubation in the refrigerator, the plates were grown under white light on either a long day photoperiod (16 hours light/8 hours dark) or short day photoperiod (8

hours light/16 hours dark) for two weeks.

2.3.3 Growing *Arabidopsis thaliana* plants on soil

Arabidopsis small plants from the plates were moved onto pots or trays containing compost soaked in a 0.2 g/l solution of the insecticide Intercept (Scotts UK). Plants were added to the top of soil and under white light on either a long day photoperiod (16 hours light/8 hours dark) or short day photoperiod (8 hours light/16 hours dark).

2.4 DNA & RNA preparations

2.4.1 Transformation of *E.coli* competent cells

Competent *E. coli* cells were placed on ice to thaw for ten minutes. Approximately 5 μl of plasmid DNA or DNA ligation reaction was added to the competent cells which were then incubated on ice for 20 minutes. The cells and DNA were heat-shocked in a waterbath at 42°C for 35 seconds then immediately placed on ice. After 2 minutes incubation on ice, 700 μl of LB media was added to the cells and DNA which were then incubated at 37°C for 1 hour with shaking at 200 rpm. Transformations were plated out on LB agar plates with appropriate antibiotic and incubated overnight at 37°C until colonies developed.

2.4.2 Purification of plasmid DNA

Plasmid DNA purification from *E. coli* was performed using Qiagen Plasmid MiniPrep kits. Cells from a single bacterial colony were inoculated into a 10 ml overnight LB medium culture with appropriate antibiotics and grown at 37°C with shaking at 200 rpm. Bacterial culture (5 ml) was pelleted by centrifugation at 3000 g for 10 minutes and supernatant was discarded. Cell lysis and DNA purification was carried out according to the manufacturer's instructions. The purified plasmid DNA was eluted with dH$_2$0 in a final volume of 50 μl. Plasmid DNA was stored at –20°C.

2.4.3 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis is a method used to separate DNA or RNA molecules according to the size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). 0.8-1% agarose gels were prepared in 1*TEAE buffer (40 nm Tris, 1 mM ETTA-Na$_2$-salt, 20 mM Acetic Acid, pH 8.5). The gel was prepared by heating the solution in the microwave until fully dissolved. To visualize the band, the agarose liquid was mixed with SYBR SAFE (Invitrogen SKU#S33102) of a final concentration 1:10.000 and the gel poured in a casting tray. A 1 kb DNA ladder was run alongside the samples. 6×loading dye (0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 30 % (w/v) glycerol) were added to the samples, before they were transferred to the gel and run at 100 V until the gel reach the middle line. The gel was analyzed under UV light and recorded using BIO-RAD Gel Doc 2000 apparatus.

2.4.4 DNA extraction and purification from agarose gel

DNA bands were separated by electrophoresis in agarose gels and bands of the expected size were excised on a UV illuminator. DNA was extracted and purified using the Qiaquick Gel Extraction Kit in accordance with the manufacturer's instructions. Purified DNA was eluted in 50 µl dH$_2$0.

2.4.5 DNA ligation

DNA obtained from PCR amplification or restriction digest was ligated in a final volume of 10 µl. Aliquots of plasmid and insert DNA were examined on an agarose gel to establish their relative concentrations. Typically insert and vector fragments were mixed in a 5:1 ratio (500 ng: 100 ng) with 2× ligation buffer (Promega), 1 unit of T4 DNA ligase (Promega) and sterile water to a final volume of 10 µl. The ligation mix was incubated at 22°C for 20 hours. Typically, 5 µl of ligation mix was used for transformation of *E. coli* cells.

2.4.6 Restriction Endonuclease digest of plasmid DNA

Plasmid DNA was digested with restriction enzymes either for analysis (20 µl reaction) or in preparation for cloning (50 µl reaction). Analytical restriction digests were performed on 7 µl of plasmid DNA in a 20 µl reaction volume with 5 - 8 units of each enzyme. Preparative restriction digests were performed on 20 µl of plasmid DNA in a 50 µl reaction volume with 10-15 units of each enzyme. When sequential preparative digests were performed, initial 50 µl digests were diluted 2 fold into secondary digest reactions (ie. 25 µl initial digest into 50 µl secondary digest). All enzymes and 10 x buffers were supplied by Roche and digest reactions were incubated at the appropriate temperature for 2-4 hours.

2.4.7 Quantification of DNA

Purified plasmid DNA concentration was assessed by absorbance measurment at 260 and 280 nm. DNA samples were diluted 50 fold in dH_2O (2 µl in 100 µl), transferred to a quartz cuvette and absorbance at 260 and 280 nm were measured against a dH_2O blank sample.

Plasmid DNA concentration in ng/µl was calculated by the following formula:

$$\text{Plasmid DNA (ng/µl)} = (OD_{260} \times 50) \times \text{Dilution Factor}$$

The ratio of 260/280 nm absorbance values indicated the purity of the samples (optimal purity being 260/280 =1.8) (Sambrook and Russel, 2001).

2.4.8 Polyadenylation of PCR products

Blunt ended PCR products produced by Phusion polymerase were polyadenylated to allow ligation into pGEM-T Easy vector. Polyadenylation reactions contained 8 µl of PCR product, 2 mM dATP, 10×Thermopol Taq polymerase buffer (NEB) and 0.5 units Thermopol Taq polymerase (NEB) in final volume of 10 µl. Reactions were incubated at 72°C for 30 minutes, chilled on ice then transferred directly to DNA ligation (typically 7 µl polyadenylation reaction in a 10 µl ligation).

2.4.9 pENTR D-TOPO based DNA ligation

PCR products destined for ligation into pENTR D-TOPO (Invitrogen) were amplified with appropriate primers containing CACC in the 5' primer termini. Purified PCR products (typically 500 ng DNA) were ligated into pENTR D-TOPO using TOPO directional cloning based on the manufacturer's instructions. Ligation reactions contained 4 μl PCR product, 1 μl salt solution (Invitrogen), 1 μl D-TOPO vector (Invitrogen) and dH_20 to a final volume of 6 μl. D-TOPO ligation reactions were incubated at 22°C for 30 minutes then transformed (typically 5 μl ligation) into *E. coli* (Section 2.4.1).

2.4.10 Gateway recombination based cloning

DNA fragments were cloned into pENTR D-TOPO or pENTR4 entry vectors to facilitate Gateway® recombination based cloning into a variety of destination vectors. Recombination reactions contained 5 μl (500 ng) entry plasmid, 1 μl destination vector, 2 μl LR clonase enzyme mix (Invitrogen) and 2 μl dH_20. Reactions were incubated at 22°C for 1 hour then inactivated by the addition of 1 unit Proteinase K (Invitrogen) for 10 minutes at 37°C. Recombination reactions (typically 5 μl) were transformed into *E. coli* (Section 2.4.1).

2.4.11 DNA sequencing

Sequencing of DNA was carried out by the Functional Genomics facility (University of Glasgow) in accordance with their instructions. Sequencing was carried out on plasmid DNA to verify sequence insert integrity of all experimental constructs genererated for this study.

2.4.12 Isolation of Genomic DNA from *Arabidopsis* plants

Three medium sized leaves were taken from plants. The leaf samples were wrapped with aluminum foil and frozen in liquid nitrogen. Using a pestle and mortar, they were crushed over the liquid nitrogen. The powder was transferred into three 1.5 ml sterile eppendorf tubes, each containing a 0.5 ml. 500 μl of DNA extraction buffer was then added. Solution was mixed by vortexing for 10 seconds and then centrifuged in a microcentrifuge [eppendorf

#5417 R] at 13,000 rpm for 10 minutes. The supernatants were taken without touching the pellet and transferred to a sterile eppendorf tube. 0.5 ml of isopropanol was added and mixed by inverting several times. The samples were left at -20°C for 10 minutes to precipitate. The samples were spun by centrifugation at 13.000 rpm for 10 minutes. The supernatant was removed using a P200 pipette and the samples were dried by tapping tube upside down into a towel (DNA may be seen as a transparent film down the side of the tube and waste material is pelletted at the bottom of the tube). 0.5 ml of 70% ethanol was added to remove traces of buffer salts and the samples were again spun by centrifugation at 13.000 rpm for 5 minutes. The ethanol was removed and the sample dried. 50 µl of H_2O were added and the tubes flicked to make sure the DNA was dissolved. The DNA samples were concentrated with a speedvac for about 15 minutes at 30°C. DNA stock samples were stored in a freezer at -20°C.

2.4.13 Isolation of plant RNA

Three medium size of leaves were taken from plants. The plant samples were frozen in ~300 µl of liquid nitrogen. Using a pestle and mortar, they were crushed over the liquid nitrogen. The powder was then transferred to 2 ml eppendorf tubes and 0.7 ml of Tri reagent [Sigma #T9424] was added. The samples were vortexed until dissolved, then placed on ice for 5 minutes. 0.5 ml of chlorofom was then added (in the fume hood). The solution was mixed for 15 seconds by inverting. The samples were then centrifuged at 14.000 rpm for 20 minutes at 4°C. This caused the samples to separate with an upper aqueous layer containing RNA, white middle membrane with DNA and lower brown layer containing everything else. The top aqueous layer was removed using P100 pipette and transferred into a sterile eppendorf tube without touching the middle layer. 0.5 ml of isopropanol was added and the sample was mixed by inverting 10 times. The samples were left for 5 minutes on ice to precipitate. The samples were then centrifuged at 14.000 rpm for 20 minutes at 4°C to pellet RNA. The supernatant was removed. 1 ml of 70% ethanol was added and the sample was again spun by centrifuged at 14.000 rpm for 1 minute at 4°C. This step was repeated to reduce salt contamination. The ethanol was removed and the sample dried. The pellet was resuspended by adding 20 µl of autoclaved water. Stock samples were

stored in the freezer at – 80°C.

2.4.14 Quantification of RNA

To make cDNA from RNA, the concentration of the RNA prepared was first determined. Using 1 ml cuvette and dilution factor of 2 µl sample in 800 µl water, a spectrometer reading was taken at 260, 280, and 320 nm wavelengths. The concentration of RNA was then calculated as follows:

$$\text{Concentration of RNA (µl / ml) } = \frac{260\text{Abs} \times 33 \times \text{dilution factor}}{1000}$$

The ratio of absorption of 260:280 should be between 1.8 and 2.0, this measure represents a test for purity (Sambrook and Russell., 2001).

2.4.15 cDNA synthesis

0.5 µg of RNA was used for each cDNA synthesis reaction. 1 µl oligo dT primer (10µM) was added and the final volume was adjusted to 10 µl with dH_2O. The solution was then incubated for 10 minutes at 70°C and was then snap-cooled on ice. Once the samples had the condensation spun down, 4µl 5x RT buffer, 2µl 0.1 DTT, 1µl Rnase inhibitor and 2µl dNTPs were added. The samples were incubated at 42°C for 2 minutes. 1 µl of RT superscript enzyme was added and mixed using a pipette tip. The sample was then incubated for a further 1.5 hours at 42°C. Afterwards, it was moved to 72°C for a further 10 minutes. After a brief centrifugation, 50 µl of water was added to each sample.

2.5 PCR methods

2.5.1 Oligonucleotide primer design

Primer oligonucleotides were either designed *de novo* or using Primer3 software (Rozen and Skaletsky, 2000) as appropriate. Typically, Primer3 designed oligonucleotides were 21 bp in length with a melting temperature (T_M) of 60°C and GC content ≥ 40%. Primers were synthesised by MWG and supplied as 100 µM stocks. Primers used in this study are listed in table 2.8.

2.5.2 Amplification of DNA by Polymerase Chain Reaction

Polymerase chain reactions (PCR) were set up using a MJ Research DNA Engine PTC-200 Peltier Thermal Cycler (Genetic Research Instrumentation, Essex, UK). PCR reactions were completed in a final volume of 20 µl. Template DNA (0.2-0.01 ng) was added to 1 x Thermopol buffer (NEB) with 0.5 µM of each primer, 250 µM dNTPs and 1 unit of *Taq* DNA polymerase (NEB).

PCR Annealing temperature (T_A) was calculated using the following formula:

$$T_A = (2 \times (A + T) + 4 \times (G + C)) - 5$$

Amplification was performed using a suitable number of cycles after an initial denaturation step of 2 minutes at 94°C. A typical cycle consisted of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute per Kb of DNA in the target amplicon. This basic program was modified as required to use specific DNA templates or primers. PCR for cloning applications was completed using proofreading Phusion DNA polymerase (NEB) in accordance with the manufacturer's instructions. Amplification was performed using a suitable number of cycles following an initial denaturation for 30 seconds at 98°C. A typical cycle consisted of denaturation at 98°C for 15 seconds, annealing at 58°C for 20 seconds and extension at 72°C for 30 seconds per Kb of DNA in the target amplicon.

2.5.3 Semi-Quantitative Reverse Transcriptase PCR

cDNA for semi-quantitative RT-PCR was prepared using Superscript II reverse transcriptase (Section 2.4.15). PCR amplification of cDNA for semi-quantitative RT-PCR measurement of mRNA was completed in a final volume of 20 μl using *Taq* DNA polymerase (Section 2.5.2). cDNA samples were checked by PCR using appropriate *Actin* primers with 2 μl cDNA and sufficient cycles (typically 24) to achieve product amplification in the linear range (Sambrook and Russel, 2001). PCR samples (16 μl) were resolved on TAE agarose gels (Section 2.4.3) and cDNA content in each PCR reaction was then normalised by comparative analysis based on *Actin* amplification. cDNA volumes were adjusted based on initial *Actin* analysis and the *Actin* PCR was repeated to check the normalisation of cDNA content in each reaction. cDNA normalisation PCRs using *Actin* primers were repeated until all samples were suitably equalised. RT-PCR to investigate a particular gene of interest was completed on normalised cDNA samples with an appropriate number of cycles.

2.6 Plant pathology methods

For analysis during pathogen infection, virulent and avirulent strains of *Pseudomonas syringae* pv. *tomato* (*Pst*) were used to infect 5 – 6 week old *Arabidopsis* plants grown under short day conditions. Bacterial strains: *P. s.* pv. *tomato* DC3000, and *P. s.* pv. *tomato* DC3000 *avrRpt2* were grown in overnight cultures of Kings media with rifampicin (50 μg/ml) and kanamycin (50 μg/ml) as described by Katagiri *et al* (Katagiri et al., 2002). Bacterial cultures were resuspended in 10 mM $MgCl_2$ and adjusted to a a final optical density (OD_{600}) of 0.2 (which corresponds to 10^8 cfu/ml). Silwett were added in final concentration 0.04%. The bacterial suspension was sprayed on the plants until all leaves covered with the pathogen suspension. The trays were then covered for a few days before. Leaves were harvested. Leaves discs from two different leaves were ground in 10mM $MgCl_2$ with a microfuge tube glass pestle. After grinding of the tissue, the samples were thoroughly vortex-mixed and diluted 1:10 serially. Samples were finally plated on Kings B medium supplemented with the appropriate antibiotic. Plates were placed at 28°C for 2 days, after which the colony-forming units were counted.

2.7 Transient expression

2.7.1 Transformation of competent *Agrobacterium* cells by electroporation

Competent cells of *Agrobacterium* strain GV3101 were thawed on ice. 1 μl (100-200 ng) of plasmid DNA was added to the cells and incubated on ice for 5 minutes. The cells containing DNA were transferred to an electroporation cuvette and pulsed with 1800V using an electroporator. 1 ml of LB medium was added immediately to the cells which were then transferred to an eppendorf and incubated for 2 hours at 28°C with shaking (200 rpm). Transformed cells were plated out on LB agar containing rifampicin (100 μg/ml) and antibiotics appropriate for the transformed plasmid. Plates were incubated at 28°C for two days until colonies developed.

2.7.2 Transient expression of gene constructs in *Nicotiana* species

Gene constructs were transiently expressed in *N. tabacum* and *N. benthamiana* plants using *Agrobacterium* mediated transformation. Cells from a single colony of recently transformed *Agrobacterium* were inoculated into an overnight 10 ml culture of LB media with rifampicin (100 μg/ml) and kanamycin (50 μg/ml) which was grown at 28°C with shaking at 200 rpm. Following overnight growth, cells were pelleted by centrifugation at 3000 g for 10 minutes then resuspended in 10 mM $MgCl_2$. Cultures were adjusted to OD_{600} 0.5 unless otherwise stated and acetosyringone was added to a final concentration of 150 μM prior to a 2 hour incubation at 22°C. Cultures were infiltrated into the abaxial leaf surface by pressure inoculation using a blunt 1 ml syringe. Following culture inoculation, plants were incubated at 22°C for 2 or 3 days before experimental analysis

2.8 Protein expression and purification from *E.coli*

2.8.1 Protein Expression

A single colony from transformed BL21 (de3) *E.coli* cells was used to inoculate 10 ml of LB medium with appropriate antibiotic and grown overnight at 37°C in an orbital shaker rotating at 200rpm. The overnight cultures were used to inoculate fresh LB medium at a dilution rate of 1:100. The cultures were incubated at 37°C in an orbital shaker rotating at 200rpm. The cultures were induced with 1M Isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD_{600} of 0.650 for a final concentration of 1 μM. The cultures were allowed to grow at the same conditions for 2 hours after which they were harvested by centrifuging at 4500g. The protein expression in the harvested cells was analysed by performing SDS-PAGE.

2.8.2 Purification of His· tag recombinant proteins.

The pellet recovered from the induced culture was resuspended in Bugbuster (Novagen) along with benzonase nuclease (Novagen) and protease inhibitors (Roche) according to the manufacturer's instructions. The soluble protein was collected by centrifuging the solution at 16.000g for 10 minutes at 4°C. The supernatant was transferred into a fresh tube and stored on ice. The column for purifying the 6× His tagged fusion protein was clamped onto a stand and 1 ml of His- resin pippeted into the tube. The resin was allowed to settle down and once settled the valve was opened to drain off the residual liquids in the column. The His resin was charged by washing the column with 1× charge buffer (50mM NiSO4) followed by a wash with 1×Binding Buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) and 1× Wash Buffer (0.5 M NaCl, 60 mM imidazole, 20mM Tris-HCl, pH 7.9). The soluble protein was loaded onto the column and the residual liquid allowed to drain off. The column was washed with 1x binding buffer followed by 1× wash buffer. The protein was eluted from the column by using 1× elute buffer (0.5 M imidazole, 0.25 M NaCl, 10 mM Tris-HCl, pH 7.9) in 1 ml fractions. The concentrations of the fractions were quantified by Bradford assay. The eluted samples were dialysed against 1x Tris buffer saline (150 mM NaCl, 10 mM Tris, pH8.0) containing 0.1 mM DTT. The dialyzed samples stored at -80°C in 50 µL. The dialyzed samples were analyzed by SDS-PAGE and western blot.

2.9 Protein methods

2.9.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and sample preparation.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis is used to separate proteins according to their size. In SDS gel electrophoresis, proteins are denatured by SDS to form individual polypeptide chains and then they are forced through a gel by an electric field. To prepare the sample, 1 ml culture of known optical density was centrifuged at 13000 g in a micro centrifuge tube. The supernatant was discarded and the cells were lysed by adding 100 μl of Bugbuster (novagen) containing 0.1 μl of Benzonase nuclease (Novagen). The lysate was made up to 300 μl using water. A 50 μl gel sample of total cell lysate was collected at this stage. The contents of the microcentifuge tube were centrifuged at 13000 g for 10 minutes. A second sample of 50 μl was collected from the supernatant (soluble fraction). The pellet was resuspended in 150 μl of water and a third gel sample 50 μl was collected (insoluble fraction). The gel samples were mixed with 4× SDS loading buffer and boiled at 94 °C for 5 minutes. Electrophoresis was performed at 80 volts, for approximately 3 hours.

2.9.2 Coomassie blue staining

Coomassie blue (also known as Brilliant blue G-250) is a blue dye commonly used in SDS-PAGE. The gel was soaked in dye for thirty minutes to overnight and then destained for thirty minutes or longer. This treatment allows the visualisation of bands indicating the presence of proteins in the gel.

2.9.3 Western blotting

Western blotting is an immunoblotting technique performed to confirm the presence of protein *in vitro*. To confirm protein expression the total, soluble and insoluble fractions were resolved on a SDS-PAGE and transferred on to a poly(vinylidene difluoride) membrane for 1 hour at 100v. The membrane was blocked with 5% milk in TBST for 1 hour. After blocking, primary antibodies were incubated with the membane at the dilutions indicated in Table 2.7. The membrane was then washed 5 times for 5 minutes in TBST before

secondary antibodies were added at the dilutions indicated in Table 2.2 for 1 hour. All secondary antibodies used in this study were horseradish peroxidase (HRP) conjugates and were developed using Millipore chemiluminescent substrate in accordance with the manufacturer's instructions.

2.10 *In vitro* Ubiquitination assay

Ubiquitination assays were performed in vitro to analyse the ubiquitin binding properties of ubiquitin cascade enzymes. The assays are performed by adding 5 mM ATP, 50 μM Ubiquitin, 100 μl/mL IPP (isopentenyl pyrophosphate), 50 mM DTT (Dithiothreitol), Ubiquitin buffer, 100 nM E1 enzyme, UBC E2 Proteins and the E3. The reactions were incubated for 1 hour at 37 °C. The reactions were quenched by adding 2× non reducing buffer and analysed by SDS-PAGE and western blotting as described in section 2.9.4.

2.11 Confocal Microscopy

The subcellular localisation of GFP was visualised using a confocal laser scanning microscope (Zeiss LSM 510) under water with a 40 x objective lens. GFP tags were excited using an argon laser at 488 nm. GFP emission was collected between 505-530 nm to avoid cross-talk with chloroplast autofluorescence.

2.12 Geldoc

All the agarose gel data in this were analyzed under UV light and recorded using BIO-RAD Gel Doc 2000 apparatus.

2.13 Sequence Alignment analysis

Alignment based analysis and sequence database interrogation was performed using the BLAST algorithm as described by Altschul et al (Altschul et al., 1997). Protein and nucleotide sequence queries were performed against genome and EST sequence repositories at the TAIR (http://www.arabidopsis.org) and TIGR (http://www.tigr.org) websites using BLASTP and BLASTN programs with default search parameters.

CHAPTER 3

An investigation into the role of UBP12 and UBP13 in plant disease resistance and cell death

3.1 Introduction

The ligation of Ub to substrates is a reversible process and all the known peptide linkages made between Ub moieties are efficiently cleaved by deubiquitinating enzymes (DUBs). Plants, animal and yeast contain numerous genes predicted to encode DUB (Vierstra, 2003). The largest subclass of DUBs is Ubiquitin Specific Proteases (UBP) (Doelling et al., 2001). Yeast (*Saccharomyces cereviceae*) genome is shown to encode 16 distinct UBP genes, where as *Arabidopsis thaliana genome* is shown to encode 27 UBP genes (Yan et al., 2000) indicating that UBPs in plants may have a more varied substrate base. In *Arabidopsis* the most complete analysis of plant UBP enzyme encoding genes was reported by Yan et al., 2000.

It has been previously reported that there is a major functional redundancy between two paralogous *Arabidopsis* UBP enzymes: AtUBP12 and AtUBP13 (Ewan., 2008). On the basis of recently reported microarray data examining pathogen associated signalling in *Nicotiana benthamiana* (Kim et al., 2006), the potential role of Arabidopsis genes *AtUBP12* and *AtUBP13* in disease resistance signalling was investigated.

This chapter reports a successful transgenic RNA interference approach used to knockdown both *AtUBP12* and *AtUBP13* transcripts. The *Arabidopsis-Pseudomonas* interaction was employed to examine the transcriptional response of *AtUBP12* and *AtUBP13* during pathogen infection, the silenced line of AtUBP12 and *AtUBP13* was isolated and alterations in their resistance to virulent strains of *Pseudomonas* were assessed. Furthermore a transient over expression approach was used to investigate possible gain of function phenotypes associated with *NtUBP12* (tobacco homologue of *AtUBP12* and AtUBP13) activity during Cf-9 triggered HR in tobacco.

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3.2 RNA interference based silencing of *AtUBP12* and *AtUBP13*

3.2.1 *AtUBP12* and *AtUBP13* specific RNA interference in *Arabidopsis* using pHELLSGATE

Reverse genetic approaches to study gene function in *Arabidopsis* rely primarily on insertional mutagenesis approaches based on T-DNA insertion. However, the use of T-DNA insertion has associated limitations when studying duplicated genes. Duplicated genes often exhibit functional redundancy that invalidates the T-DNA approach as the potential phenotype of a null allele is obscured by the presence of a functional sibling (Gu et al., 2003).

RNA silencing known as RNA interference (RNAi) and Post Transcriptional Gene Silencing (PTGS) in plants are nucleotide sequence homology based approaches that can be used to knock-down the expression of known target genes (Mansoor et al., 2006). A common feature of gene specific RNAi is the target double stranded RNA (dsRNA), which is recognized by a processing enzyme (Dicer) that cleaves it into small (21-25 nucleotides) fragments. These small RNAs are then incorporated into a complex known as the "RNA-induced silencing complex" (RISC). RISC then degrades single stranded RNA (ssRNA) including mRNA of target genes in a sequence specific manner (Mansoor et al., 2006).

Various approaches have been used to produce dsRNA in plants. Initially this was achieved by transforming plants separately with constructs to produce sense and antisense RNA and subsequently crossing these to induce dsRNA formation (Waterhouse, 1998). Presentation of dsRNA in the plant cell can occur from naturally occurring viral RNA (the replication of which produces dsRNA). This principle is commonly applied in Virus Induced Gene Silencing (VIGS) to initiate transient gene silencing in virus compatible host plant species (Ratcliff et al., 2001). The other approach is the generation of 'hairpin' RNA, which is made by expressing sense and antisense cDNA sequences of target gene, separated by an intron and transcribed under the same promotor. Upon transcription, the complimentary sequences hybridise and form hairpinRNA (hpRNA) molecules resulting in dsRNA production and hence gene silencing (Smith et al., 2000) (Mansoor et al., 2006).

This hairpin RNAi approach was utilised to address potential functional redundancy between *AtUBP12* and *AtUBP13*. The generation of hairpin RNA expressing constructs has been simplified by the recent development of the Gateway compatible pHELLSGATE vector (Helliwell and Waterhouse, 2003). The pHELLSGATE vector allows recombination based transfer of DNA fragments from attL1-attL2 sites in a Gateway entry clone into pHELLSGATE12 which carries two attR1-attR2 cassettes under a CaMV 35S promoter (Helliwell and Waterhouse, 2003). The attR1-attR2 cassettes are separated by an intron and are in opposite sense orientations with respect to the promoter giving rise to inverted repeat constructs. Expression of gene fragments transferred to pHELLSGATE *in planta* produces a hairpin RNA with the intron spliced out. This hpRNA will present the requisite dsRNA signal to initiate gene silencing targeted against host gene (or genes) corresponding to the pHELLSGATE expressed cDNA fragment.

3.2.2 RNAi based silencing of *AtUBP12* and *AtUBP13*

In this study, RNAi silencing of *AtUBP12* and *AtUBP13* was initiated by Richard Ewan (PhD student within the laboratory of Dr. Ari Sadanandom). A pHELLSGATE12 construct was generated using a cDNA fragment from *AtUBP13* which would generate siRNAs with homology to *AtUBP12* and initiate silencing of both genes (designated construct – UBP_RNAi).

420 bp regions unique to *AtUBP12* and *AtUBP13* were selected from the cDNA sequence of *AtUBP13* (Figure 3.1 A). Alignment analysis of the selected *AtUBP13* cDNA fragment indicated multiple regions over 21 nucleotides in length with continuous identity to *AtUBP12* (Figure 3.1 B) indicating it could initiate silencing of both genes. To assess potential off target silencing effects, the selected UBP_RNAi cDNA fragment was compared with a library of Arabidopsis genome using Basic Local Alignment Search Tool (BLAST from TAIR website) and recovered *AtUBP13* and *AtUBP12* as top scoring matches (E-values of 0 and $4e^{-84}$ respectively) followed by a low scoring third non-UBP match to *AtMAP65-1* (E-value 0.023). There were no additional UBP genes recovered using this cDNA fragment and the extent of continuous sequence match to *AtMAP65-1* was limited to a single region of 13 base pairs. This *in silico* sequence analysis indicated that the cDNA region selected for the

UBP_RNAi construct was unlikely to cause 'off target' cross silencing effects and at the same time generate the required sequence specific-dsRNA required for silencing both *AtUBP12* and *AtUBP13* gene expression.

The UBP_RNAi cDNA fragment was amplified from *Arabidopsis* cDNA using Gateway compatible primers (UBP_RNAi_5 and UBP_RNAi_3) and TOPO cloned into the appropriate Gateway entry vector (pENTR D-TOPO). Using Gateway recombination, the UBP_RNAi fragment was transferred to pHELLSGATE12 plant transformation vector resulting DNA construct UBP_RNAi pHELLSGATE12. This DNA construct was used to transform *Arabidopsis* wild type (Col-0) plants (Ewan, 2008). The T_0 and T_1 of UBP_RNAi were selected by Richard Ewan and I carried forward further analysis on 26 independent T_1 UBP_RNAi transgenic seeds.

(A).

AtUbp12

AtUbp13

(B).

```
              1         10        20        30        40        50        60        70        80        90        100       110       120       130
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AtUbp12   CCTGGATTTACGTCCTATTCCTCCTCCTGAAAAATCAAAAGAAGATATTCTTCTTTTCTTCAAGCTTTATGACCCCGAGAAGGCAGTATTAAGCTATGCTGGCAGGCTGATGGTGAAAAGTTCCAGTA
AtUbp13   GGACCGGATGACCTTCCTATTCCTCCCCCAGAAAAAACTTCTGAGGATATCCTTCTTTTCTTTAAACTCTATGACCCTGAGAACGCAGTACTAAGATATGTTGGCAGGCTAATGGTGAAAAGTTCCAGTA
Ubp_RNAi  GGACCGGATGACCTTCCTATTCCTCCCCCAGAAAAAACTTCTGAGGATATCCTTCTTTTCTTTAAACTCTATGACCCTGAGAACGCAGTACTAAGATATGTTGGCAGGCTAATGGTGAAAAGTTCCAGTA
Consensus ggaCcGGATgacCtTCCTATTCCTCCcCCaGAAAAAaCttctGAggGATATcCTTCTTTTCTTtAAaCTcTATGACCCtGAGAAcGCAGTACTAAGaTATGtTGGCAGGCTaATGGTGAAAAGTTCCAGTA

              131       140       150       160       170       180       190       200       210       220       230       240       250       260
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AtUbp12   AGCCTATGGATATAACTGGAAAACTGAATGAAATGGTTGGCTTTGCTCCTGATGAAGAAATAGAACTTTTTGAGGGAAATCAAGTTTGAACCTTGTGTTTATGTGCGAACACTTGGATAAGAAAACTTCATT
AtUbp13   AGCCCATGGATATAGTAGGGCAATTGAATAAAATGGCTGGTTTTGCTCCTGATGAGGAAATAGAACTTTTTGAGGGAAATAAAGTTTGAACCTTGCGTAATGTGTGAACAGATTGATAAGAAGACTTCTTT
Ubp_RNAi  AGCCCATGGATATAGTAGGGCAATTGAATAAAATGGCTGGTTTTGCTCCTGATGAGGAAATAGAACTTTTTGAGGGAAATAAAGTTTGAACCTTGCGTAATGTGTGAACAGATTGATAAGAAGACTTCTTT
Consensus AGCCcATGGATATAgtaGGgcAAtTGAATaAAATGGcTGGtTTTGCTCCTGATGAgGAAATAGAACTTTTTGAGGGAAATaAAGTTTGAACCTTGcGTaATGTGtGAACAgaTtGATAAGAAgACTTCtTT

              261       270       280       290       300       310       320       330       340       350       360       370       380       390
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AtUbp12   CAGGATTGTGTCAAATTGAAGATGGAGATATCATTTGCTTTCAGAAACCTCTTGTTAACAAGGAGATTGAATGCCTCTACCCAGCTGTGCCTTCGTTTCTTGAATATGTCCAGAATAGACAGCTGGTCCGG
AtUbp13   CAGGCTGTGTGCAAATTGAAGATGGAGATATCATTTGTTTATCAGAAACCTCTTTCTATCGAGGAGAGTGAATTTCGATACCCAGATGTGCCATCATTTTTGGAGTATGTACAGAATCGAGAGCTGGTGCGT
Ubp_RNAi  CAGGCTGTGTGCAAATTGAAGATGGAGATATCATTTGTTTATCAGAAACCTCTTTCTATCGAGGAGAGTGAATTTCGATACCCAGATGTGCCATCATTTTTGGAGTATGTACAGAATCGAGAGCTGGTGCGT
Consensus CAGgcTGTGTGCAAATTGAAGATGGAGATATCATTTGtTaTCAGAAACCTCTTtcTAtCgAGGAGAgTGAATttCgaTACCCAGaTGTGCCaTCaTTTtTgGAgTATGTaCAGAATcGAgAGCTGGTgCGt

              391       400       410       420       430       440       450       460       470       480 482
              |---------+---------+---------+---------+---------+---------+---------+---------+---------+----------+-|
AtUbp12   TTTCGTGCTCTGGAAAAACCTAAAGAAGATGAGTTTGTTCTGGAGTTGTCGAAGCAGCACACTTATGACGATGTTGTGGAGAAAGTGGCTGA
AtUbp13   TTTCGCACACTGGAAAAACCAAAAGAGGATGAGTTTACTATGGAGCTGTCAAAGCTGCACACTTATGATGATGTAGTGGAAAGGGTGGCTGA
Ubp_RNAi  TTTCGCACACTGGAAAAACCAAAAGAGGATGAGTTTACTATGGAGCTGTCAAAGCTGCACACTTATGATGATGTAGTGGAAAGGGTGGCTGA
Consensus TTTCGcaCaCTGGAAAAACCaAAAGAgGATGAGTTTacTaTGGAGcTGTCaAAGCtGCACACTTATGAtGATGTaGTGGAaAggGTGGCTGA
```

Figure 3.1 Selection of a cDNA fragment to initiate hpRNAi gene silencing of *AtUbp12* and *AtUbp13*

Hairpin based RNAi silencing of *AtUbp12* and *AtUbp13*, (A) Domain diagrams of *AtUbp12* and *AtUbp13*, red box indicates *AtUbp12* and *AtUbp13* cDNA selected for UBP_RNAi silencing construct. (B) Sequence alignment of UBP_RNAi cDNA fragment from *AtUbp13* and corresponding region in *AtUbp12*. Alignment colour indicates high conservation (red), low conservation (blue), no conservation (black).

To ascertain silencing of *AtUbp12* and *AtUbp13*, seeds from the 26 individual T_1 lines were grown on kanamycin selective plates. The kanamycin T_2 resistant seedlings from each line were transferred to soil and grown for further analysis.

3.2.3 RT-PCR Analysis of T_2 UBP_RNAi plants

Co-silencing of *AtUBP12* and *AtUBP13* genes in the T_2 UBP_RNAi plants was assessed by RT-PCR. Total RNA was extracted from rosette leaves of 4 week old T_2 UBP_RNAi plants and wild type plants (Col-0) was used as control, then the relative expression of *AtUBP12* and *AtUBP13* mRNA was assessed by RT-PCR using primers specific for *AtUBP12* (AtUBP12_C207S.3 and AtUB12_GW.5) or *AtUBP13* (AtUBP13_5 and AtUBP13_3). *AtUBP12* and *AtUBP13* RT-PCR amplicons were selected from cDNA regions outside the UBP_RNAi fragment to ensure amplification from endogenous mRNAs rather than the overexpressed transgenic UBP_RNAi fragment (figure 3.2A).

Multiple T$_2$ UBP_RNAi lines were analysed as the efficiency of hpRNAi has been reported to vary significantly between different transgenics (Helliwell and Waterhouse, 2003). RT-PCR analysis of the 26 independent UBP_RNAi lines demonstrated that a clear reduction in mRNA levels of both *AtUBP12* and *AtUBP13* relative to Col-0 control only in two lines (line #23 and line #26) (figure 3.2B).

(A).
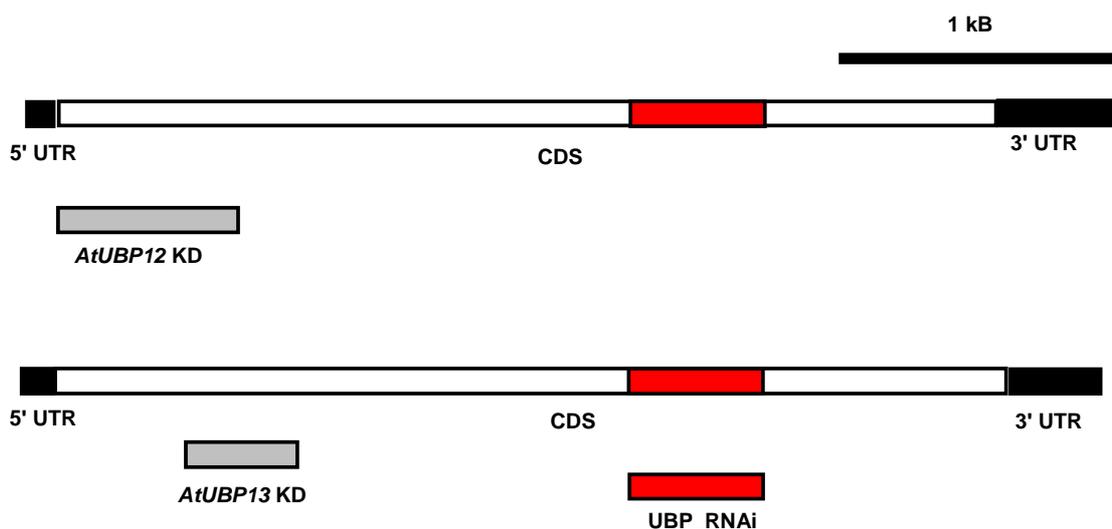
(B)
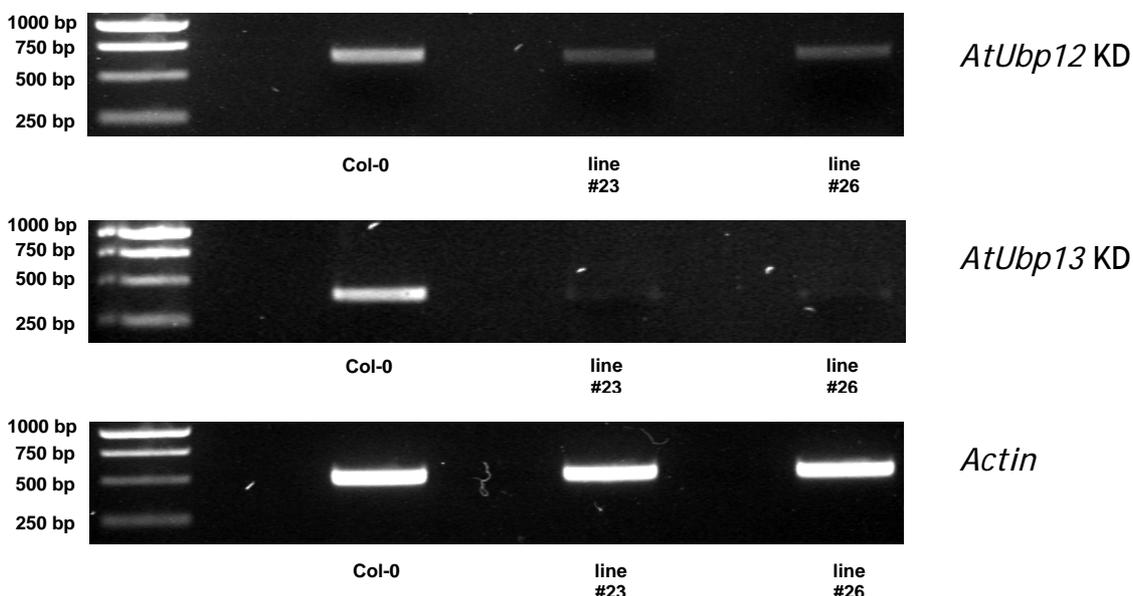
Figure 3.2 Expression analysis of *AtUbp12* and *AtUbp13* in *Arabidopsis* UBP_RNAi T$_2$ generation.

RT-PCR analysis of *Arabidopsis* UBP_RNAi T$_2$ plants. Domain diagrams of *AtUbp12* and *AtUbp13* (A) indicating the respective locations of RT-PCR amplicons (grey boxes - *AtUbp12* KD and *AtUbp13* KD) amplified to analyse gene silencing and cDNA fragment (red box) selected for UBP_RNAi silencing construct. (B) RT-PCR showing clear reduction in mRNA levels of both AtUbp12 and *AtUbp13* in line #23 and line #26.

Having identified transgenic plants with efficient co-silencing of *AtUBP12* and *AtUBP13* in the T_2 generation, 2 individual T_2 plants from UBP_RNAi lines #23 and #26 were grown to set T_3 seed. T_3 seed lines of UBP_RNAi were identified on kanamycin selective plates and confirmed silencing line were grown for further characterization.
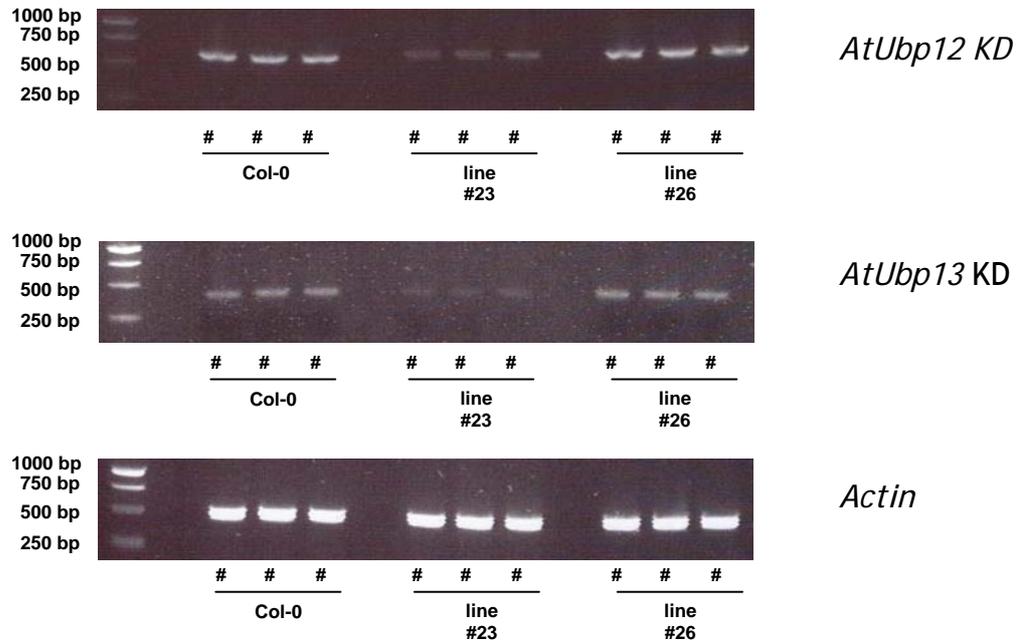
Figure 3.3 Expression analysis of *AtUbp12* and *AtUbp13* in *Arabidopsis* UBP_RNAi T_3 generation.

RT-PCR analysis of *Arabidopsis* UBP_RNAi T_3 plants. RT-PCR showing clear reduction in mRNA levels of both *AtUbp12* and *AtUbp13* in line #23 only.

RT-PCR analysis of *Arabidopsis* UBP_RNAi T_3 plants. RT-PCR showing clear reduction in mRNA levels *AtUbp12* and *AtUbp13* in line #23.

The marked reduction in silencing efficiency indicated that line #26 expression levels was effectively the same as wild type and suggested that the expression of the transgenic was affected in later T_3 generation. However UBP silencing was still efficient in line #23 and further studies were undertaken using line #23.

3.2.4 Silencing of *AtUBP12 and AtUBP13* exhibit drastic growth retardation

In the initial stages of this study, UBP_RNAi plants were isolated isolated from several lines (Section 3.1.2). Growth of UBP_RNAi plant under long day conditions indicated no obvious morphological differences when compared to wild type plants. Interestingly, results obtained in this study demonstrate that silencing of *ubp12* and *ubp13* in *Arabidopsis* exhibit a drastic growth retardation which is particularly prevalent under short day growth conditions (Figure 3.4).

Col-0   Line #23   Line #26

Figure 3.4 Growth retardation of UBP_RNAi T$_3$ generation.

Representative picture of UBP_RNAi (line #23 and #26) and Col-0. 5 weeks plants after germination following growth under a short day photoperiod (8 hours light/16 hours dark). Silencing of both *ubp12* and *ubp13* were effective in line #23.

3.2.5 Analysis of disease resistance in UBP12&13_RNAi plants

Selected line of UBP_RNAi (line #23) were infected with virulent strains of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) to assess perturbations in disease resistance.

Short day grown plants, aged 5-6 weeks were infected by spray inoculation of high titre *Pseudomonas* strains (1 x 10^8 cfu/ml) as described by Zipfel *et al* (Zipfel et al., 2004). Infected plants were returned to short day growth conditions under high humidity and bacterial growth was measured after 3 and 4 days by colony counting (Figures 3.5).

Bacterial growth measurements were based on six independent replicates (Zipfel et al., 2004). Colony count data indicated significant difference in resistance between Col-0 control and UBP_RNAi mutant alleles during virulent *Pst* DC3000 infection. At 3 and 4 days post infection, virulent *Pst* DC3000 demonstrated lower bacterial log growth on the UBP_RNAi line than Col-0. The data were shown in figure 3.7.
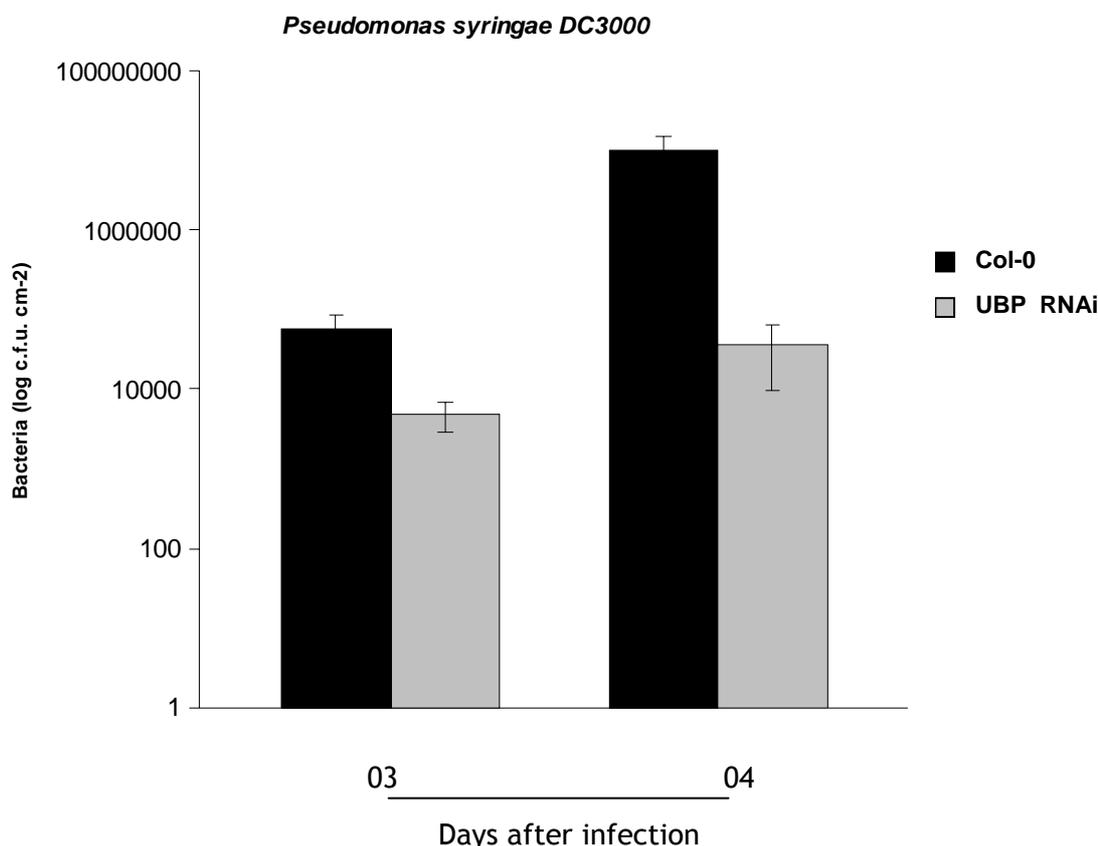
Figure 3.5 Growth assay of *Pst* DC3000 in UBP_RNAi line #23.

Bacterial growth was measured three and four days after spray inoculation, each data point represents the average and standard deviation of six replicates in logarithmic scale.

3.3 *Nicotiana tabaccum* UBP12 orthologs

Results presented in section 3.2 indicate that both *AtUBP12* and *AtUBP13* genes in Arabidopsis are involved in regulation of pathogenesis. Ewan (2008) showed that *ubp12* and *ubp13* double knock out (KO) mutants are embryo lethal. The gene silencing approach used to show that *AtUBP12* and *AtUBP13* are involved in pathogenesis.

Investigations were conducted to identify solanaceous orthologs of AtUBP12 which would facilitate UBP12 function studies in *N. tobaccum* using transient assays (Ewan, 2008). Sequence analysis of NtUBP12 indicated it shared 83% amino acid identity with AtUBP12.

```
           1        10        20        30        40        50        60        70        80        90       100       110       120       130
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AtUBP12    MTHMTPPPVDQPEDEEMLVPNSDL-VDGPAQPMEVTQPETAASTVENQPAEDPPTLKFTHTIPNFSRQNTRKHYSDVFVVGGYKHRILIFPKGNNVDHLSHYLDVSDAASLPYGHSRYAQFSLAVVNQIH
AtUBP13    MTHMTPPPLDQQEDEEMLVPNPDL-VEGP-QPMEVAQTDPAATAVENPPPEDPPSLKFTHTIPMFTRLNTRKHYSDVFVVGGYKHRILIFPKGNNVDHLSHYLDVADRANLPYGHSRYSQFSLAVVNQVN
NtUBP12    MTHMTPPPVD-PEEDEMLVPNSDFPVEGP-QPMEVATADTA-STYDGPPVDDPPSARFTHTIENFSRLNSKKLYSDVFHVGGYKHRILIFPKGNNVDHLSHYLDVADSPALPYGHSRHAQFSLAVLNRVH
Consensus  MTHMTPPPvDqpE##EMLVPNsDl.V#GP.QPMEVaq.#tAastV#npP.#DPPslkFTHTIpnFsRLNtrKhYSDVFvVGGYKHRILIFPKGNNVDHLSHYLDVaDaa.LPYGHSRyaQFSLAVvNq!h

           131      140       150       160       170       180       190       200       210       220       230       240       250       260
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AtUBP12    TRYTVRKETQHQFNARESDHGFTSFMPLSELYDPSRGYLVNDTVLVEAEVAVRKVLDYHSYDSKKETGFVGLKNQGATCYMNSLLQTLYHIPYFRKAVYHHPTTENDAPTASIPLALQSLFYKLQYNDTS
AtUBP13    NRYSIRKETQHQFNARESDHGFTSFMPLSELYEPTRGYLVNDTVLIEAEVAVRKVLDYHSYDSKKETGFVGLKNQGATCYMNSLLQTLYHIPYFRKAVYHHPTTENDAPTASIPLALQSLFYKLQYNDTS
NtUBP12    NKFTVRKDTQHQFNARESDHGFTSFMPLSELYDPIRGYLVDDTYIVEAEDYAVRRVIDYHSHDSKKETGCVGLKNQGATCYMNSLLQTLYHIPYFRKAVYHHPTTENDNPSGSIPLALQSLFYKLQYSDTS
Consensus  nr%t.!RK#TQHQFNARESDHGFTSFMPLSELY#P.RGYLV#DTVl!EA#YAVRKv1DYHSyDSKKETGfVGLKNQGATCYMNSLLQTLYHIPYFRKAVYHHPTTENDaPtaSIPLALQSLFYKLQYnDTS

           261      270       280       290       300       310       320       330       340       350       360       370       380       390
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AtUBP12    VATKELTKSFGHDTYDSFMQHDVQELNRVLCEKLEDKMKGTVVEGTIQQLFEGHHMNYIECINVDFKSTRKESFYDLQLDVKGCKDVYASFDKYVEVERLEGDNKYHAEGHGLQDAKKGVLFIDFPPVLQ
AtUBP13    VATKELTKSFGHDTYDSFMQHDVQELNRVLCEKLEDKMKGTVVEGTIQKLFEGHHMNYIECINVDYKSTRKESFYDLQLDVKGCKDVYASFDKYVEVERLEGDNKYHAEGHDLQDAKKGVLFIDFPPVLQ
NtUBP12    VATKELTKSFGHDTYDSFMQHDVQELNRVLCEKLEDKMKGTVVEGTIQQLFEGHHMNYIECINVDYKSTRKESFYDLQLDVKGCRDVYASLDKYVEVERLEGDNKYHAEKYGLQDARKGVLFIDFPPVLQ
Consensus  VATKELTKSFGHDTYDSFMQHDVQELNRVLCEKLEDKMKGTVVEGTIQqLFEGHHMNYIECINVD%KSTRKESFYDLQLDVKGCkDVYASfDKYVEVERLEGDNKYHAEghgLQDAkKGVLFIDFPPVLQ

           391      400       410       420       430       440       450       460       470       480       490       500       510       520
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AtUBP12    LQLKRFEYDFMRDTMVKINDRYEFPLELDLDREDGKYLSPDADRSVRNLYTLHSVLVHSGGVHGGHYYAFIRPTLSDQHYKFDDERVTKEDLKRALEEQYGGEEELPQTNPGFNNNPPFKFTKYSNAYML
AtUBP13    LQLKRFEYDFMRDTMVKINDRYEFPLQLDLDREDGRYLSPDADKSVRNLYTLHSVLVHSGGVHGGHYYAFIRPTLSDQHYKFDDERVTKEDVKRALEEQYGGEEELPQNNPGFNN-PPFKFTKYSNAYML
NtUBP12    LQLKRFEYDFVRDTMVKINDRYEFPLELDLDRENGKYLSPDADRSVRNLYTLHSVLVHSGGVHGGHYYAYIRPTLSDQHFKFDDERVTKEDSKRALEEQYGGEEELPHANPGFNN-SPFKFTKYSNAYML
Consensus  LQLKRFEYDFmRDTMVKINDRYEFPL#LDLDRE#GkYLSPDADrSVRNLYTLHSVLVHSGGVHGGHYYA%IRPTLSDQH%KFDDERVTKED.KRALEEQYGGEEELPq.NPGFNN.pPFKFTKYSNAYML

           521      530       540       550       560       570       580       590       600       610       620       630       640       650
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AtUBP12    VYIRESDKDKIICNVDEKDIREHLRVRLKKEQEEKEDKRRYKAQAHLYTIIKVARDEDLKEQIGKDIYFDLVDHDKVRSFRIQKQTPFQQFKEEVAKEFGVPVQLQRFHIHAKRQNHTYRPNRPLTPQEE
AtUBP13    VYIRESDKDKIICNVDEKDIREHLRVRLKKEQEEKEDKRKYKAQAHLFTTIKVARDDDITEQIGKNIYFDLVDHEKVRSFRIQKQTPFQQFKEEVAKEFGVPVQLQRFHIHAKRQNHTYRPNRPLSPNEE
NtUBP12    VYIRESDKEKIICNVDEKDIREHLRVRLKKEQDEKEQKRKEKAEAHLYTIIKVARDEDLGEQIGKDIYFDLVDHDKVRSFRIQKQMAFTQFKEEVAKELGIPVQFQRYHLHAKRQNHTYRPNRPLTPQEE
Consensus  VYIRESDK#KIICNVDEKDIREHLRVRLKKEQ#EKE#KRKkyKA#AHL%TiIKVARD#Dl.EQIGK#IYFDLVDH#KVRSFRIQKQtpFqQFKEEVAKEFG!PVQlQR%HiHAKRQNHTYRPNRPLtP#EE

           651      660       670       680       690       700       710       720       730       740       750       760       770       780
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AtUBP12    LQPVGQIREASNKANTAELKLFLEVEH-LDLRPIPPPEKSKEDILLFFKLYDPEKAVLSYAGRLMVKSSSKPMDITGKLNEMVGFAPDEEIELFEEIKFEPCVMCEHLDKKTSFRLCQIEDGDIICFQKP
AtUBP13    LQTVGQIREASNKANNAELKLFLEIERGPDDLPIPPPEKTSEDILLFFKLYDPENAYLRYVGRLMVKSSSKPMDIVGQLNKMAGFAPDEEIELFEEIKFEPCVMCEQIDKKTSFRLCQIEDGDIICYQKP
NtUBP12    TQSVGQLREVSNKANNAELKLYLEVEFGLDLRPCPPPEKTKEDILLFFKLYDPLKEEHRYVGRLFVKGSGKPLEILTKLNELAGFSPDEEIELFEEIKLDPNVMCEPIDHKLTFRGSQLEDGDIICIQKP
Consensus  1Q.VGQiREaSNKANnAELKL%LE!E.glDlrPiPPPEKtkEDILLFFKLYDPekav$rYvGRLmVKsSsKP$#I.gkLNe$aGFaPDEEIELFEEIKF#PcVMCE.iDkKtsFRlcQiEDGDIIC.QKP

           781      790       800       810       820       830       840       850       860       870       880       890       900       910
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AtUBP12    LVNKEIE-CLYPAVPSFLEYYQNRQLVRFRALEKPKEDEFVLELSKQHTYDDVYEKVAEKLGLDDPSKLRLTSHNCYSQQPKPQPIKYRGVDHLSDMLVHYNQTSDILYYEVLDIPLPELQGLKTLKVAF
AtUBP13    LSIEESE-FRYPDVPSFLEYYQNRELVRFRTLEKPKEDEFTMELSKLHTYDDVVRVAEKLGLDDPSKLRLTSHNCYSQQPKPQPIKYRGVDHLSDMLVHYNQTSDILYYEVLDIPLPELQGLKTLKVAF
NtUBP12    LRSQTSEQYRFPDVPSFLEYYHNRQVVRFRSLEKPKEDDFSLELSKQDTYDDVVERVAQRLGVDDPSKIRLTPHNCYSQQPKPQPIKYQGVDRLTEMLVHYNQTSDILYYEVLDIPLPELQCLKTLKVAF
Consensus  L...esE..r%PdVPSFLEYYqNR#1VRFR.LEKPKED#F.$ELSKqhTYDDVVErVA#kLGlDDPSK1RLTsHNCYSQQPKPQPIKYrGVDhLs#MLVHYNQTSDILYYEVLDIPLPELQgLKTLKVAF

           911      920       930       940       950       960       970       980       990      1000      1010      1020      1030      1040
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AtUBP12    HHATKEEVVIHNIRLPKQSTVGDVINELKTKVELSHPDAELRLLEVFYHKIYKIFPSTERIENINDQYHTLRAEEIPEEEKNIGPNDRLILVYHFAKETGQNQ-QVQNFGEPFFLVIHEGETLEEIKNRI
AtUBP13    HSATKDEVIIHNIRLPKQSTVGDVINELKTKVELSHQDAELRLLEVFFHKIYKIFPSTERIENINDQYHTLRAEEIPEEEKNIGPNDRLIHVYHFTKEAGQNQ-QVQNFGEPFFLVIHEGETLEEIKTRI
NtUBP12    YNSAKDEVTIHTIRLPKQSTVDDVLNHLKTKVELSHPDAELRLLEVFYHKIYKIFPPTERIEDINDQYHTLRAEEIPEEEKNLGPHDRLIHVYHFMKDTTQNQAHVQNFGEPFFLVIHEGETLTEVKARI
Consensus  h.atK#EV.IHnIRLPKQSTVgDViNeLKTKVELSHpDAELRLLEVF%HKIYKIFPsTERIE#INDQYHTLRAEEIPEEEKNiGPnDRLIhVYHF.K#tgQNQ.qVQNFGEPFFLVIHEGETLeE!K.RI

           1041     1050      1060      1070      1080      1090      1100      1110      1120
           |--------+---------+---------+---------+---------+---------+---------+---------|
AtUBP12    QKKLHVSDEDFAKHKFAFHSMGRPEYLQDTDVVYNRFQRRDVYGAFEQYLGLEHADTTPKRAYANQNRHAYEKPVKIYN
AtUBP13    QKKLHVPDEDFAKHKFASFSMGRPDYLLDTDVVYNRFQRRDVYGAHEQYLGLEHIDNAPKRAYAANQNRHAYEKPVKIYN
NtUBP12    QKKLQYPDEEFSKHKFAFLSMGRPDYLQDSDVVSNRFQRRDVYGAHEQYLGLEHADNAPKRSYASNQNRHTFEKPVRIYN
Consensus  QKKLhVpDE#FaKHKFAf.SMGRP#YLqDtDVVyNRFQRRDVYGAwEQYLGLEHaDnaPKRaYRaNQNRHa%EKPVkIYN
```

Figure 3.6 Multi Alignment of tobacco UBP12 (NtUBP12) with its *Arabidopsis* orthologs (AtUBP12 and AtUBP13).

The NtUBP12 cDNA were translated and aligned against the sequence of AtUBP12 and AtUBP13 obtained from TAIR website

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3.3.1 Cloning of NtUBP12 into Gateway Vector

We wished to investigate the sub cellular localization of NtUBP12, therefore GFP was fused to the N-terminus of NtUBP12 (Palmer and Freeman, 2004). GFP tagged fusion proteins were made using the Gateway® compatible pGWB6 (GFP tagged) destination vector. Full length *NtUBP12Wt* gene (wild type) and the mutant form of *NtUBP12Wt* which is loss UBP12 activity, *NtUBP12C208S* (*NtUBP12Wt* with amino acid substitution in the active site, position 208) were PCR-amplified by using primers incorporated with *Not*1 and *Asp*718 restriction ezymes.
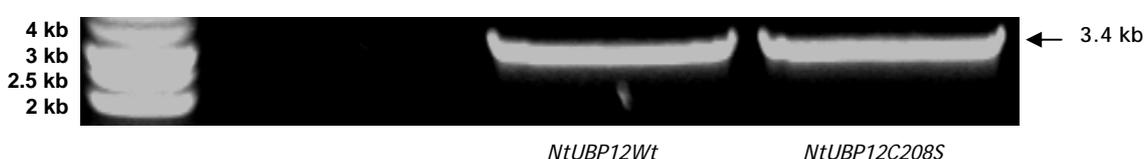
Figure 3.7 Agarose gel showing Phussion PCR of *NtUBP12Wt* and *NtUbp12C208S*.

The amplified product was cloned into pGEMT-Easy vector. The colony from the plates was checked by restriction digestion using *Not*I and *Asp*718. The size of *NtUBP12* cDNA fragment is around 3.4 kb and 3 kb for the pGEMT-Easy. Restriction digestion results indicate *NtUBP12Wt* was successfully cloned into p-GEMT easy vector in colony 6 (figure 3.8 A). *NtUbp12C208S* was also successfully cloned in p-GEMT easy vector (figure 3.8 B).
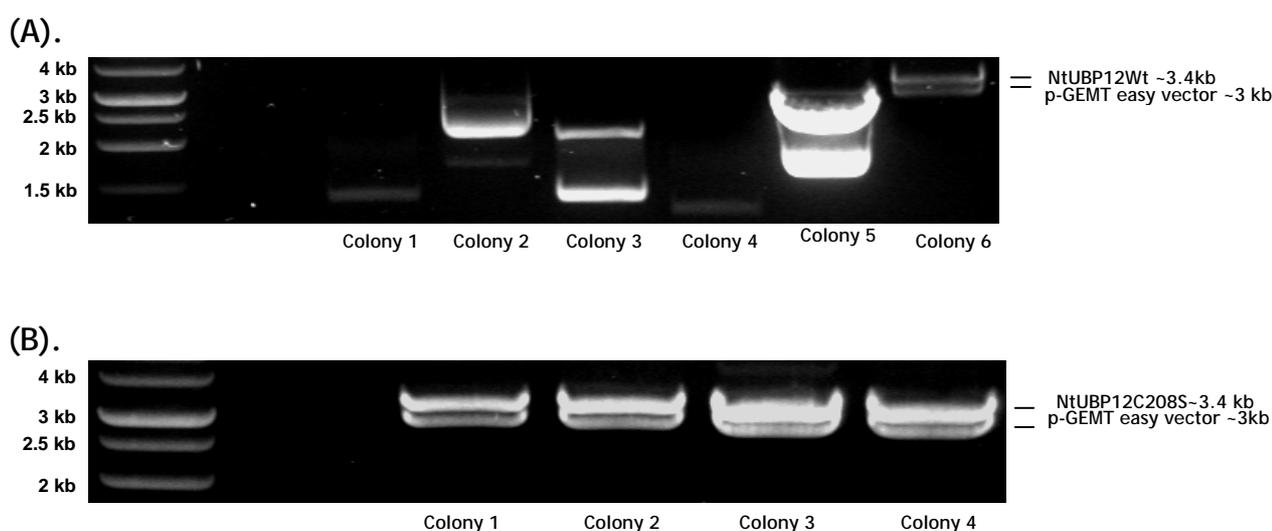
Figure 3.8 Agarose gel analysis showing restriction digestion of p-GEMT *NtUBP12Wt* (A) and p-GEMT *NtUBP12C208S* (B) with *Not*I and *Asp*718.

To generate the *NtUBP12* entry clone, the *NtUBP12Wt* and *NtUBP12C208S* cDNA were then subcloned into pENTR4 at *Not*I and *Asp*718 restriction sites. pENTR4 and *NtUBP12* cDNA were cut using *Not*I and *Asp*718 enzymes (figure 3.9).
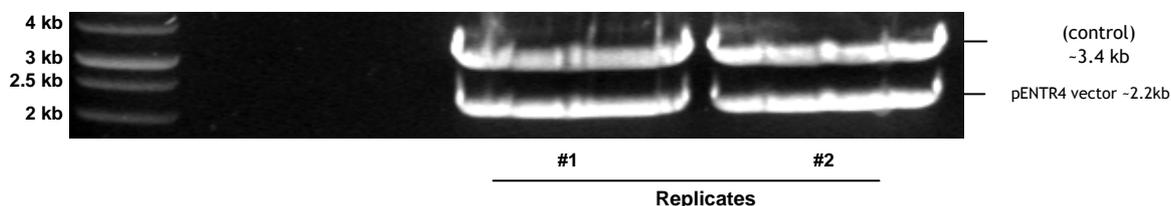
Figure 3.9 Agarose gel analysis of restriction digestion of pENTR4 vector with *Not*I and *Asp*718.

The purified fragment of *NtUBP12Wt*, *NtUBP12C208S* and pENTR4 were then ligated to generate the entry clone. Three colonies of each plate were checked by restriction digestion using the same enzymes (*Not*I and *Asp*718). All the colonies showed insertion into pENTR4 vector (figure 3.10).

(A).
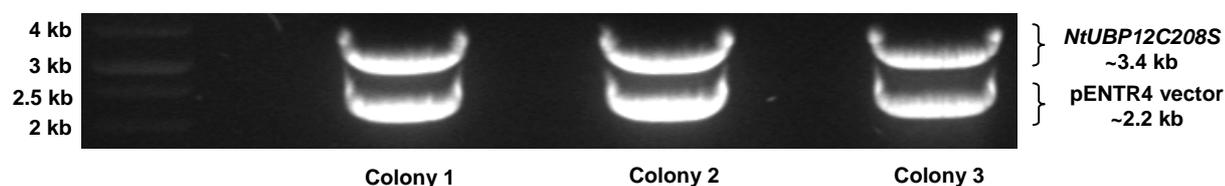
(B).

Figure 3.10 Agarose gel analysis of restriction digestion of (A) pENTR4 *NtUBP12Wt* and (B) pENTR4 *NtUBP12C208S* with *Not*I and *Asp*718.

Full length cDNA from each respective NtUBP12 entry clone was transferred into the pGWB6 destination vector by gateway LR recombination to generate N-terminal GFP tagged *NtUBP12Wt* and *NtUBP12C208S* fusion constructs.

3.3.2 NtUBP12 overexpression approaches proves that solanaceous UBP12 functions as a negative HR regulator

Using transgenic Cf-9 tobacco lines, en efficient HR response can be triggered following infiltration of extracted Avr9 peptide (Hammond Kossack et al., 1998).

Transient overexpression using *Agrobacterium* refers to Sparkes et al., 2006. *Agrobacterium* mediated transgene expression in *Nicotiana* species typically results in the accumulation of corresponding protein from two days after construct inoculation (Sparkes et al., 2006).

For transient expression studies, *Agrobacterium* cultures containing GFP-NtUBP12Wt and GFP-NtUBP12C208S fusion constructs were infiltrated into Cf-9 tobacco at OD$_{600}$ 0.5.

The results from experiments 3.11 and 3.12 indicate that the mutant form of GFP-NtUBP12Wt, GFP-NtUBP12C208S is unable to suppress cell death where as wild type protein is able to suppress cell death. The result also indicates that the cysteine residue present at the position 208 is the active site of NtUBP12Wt protein.
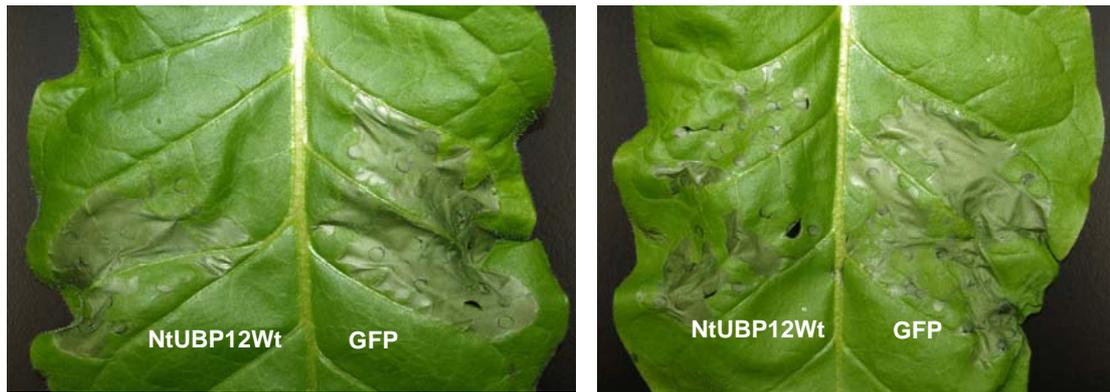
(A) 20 fold Avr9　　　　　　(B) 30 fold Avr9

Figure 3.11 Transient overexpression of NtUBP12 in Cf-9 tobacco supresses Avr9 elicited HR.

HR development in Cf-9 tobacco leaf segments overexpressing GFP-NtUBP12 (left segments) and GFP (right segments) following Avr9 elicitor infiltration. Picture taken after 4 days Avr9 elicitor infiltration.

Equivalent transient over expression assays were conducted using GFP-NtUBP12C208S active site mutant construct (figure 3.12). No HR suppression was observed during over expression of GFP-NtUBP12C208S.
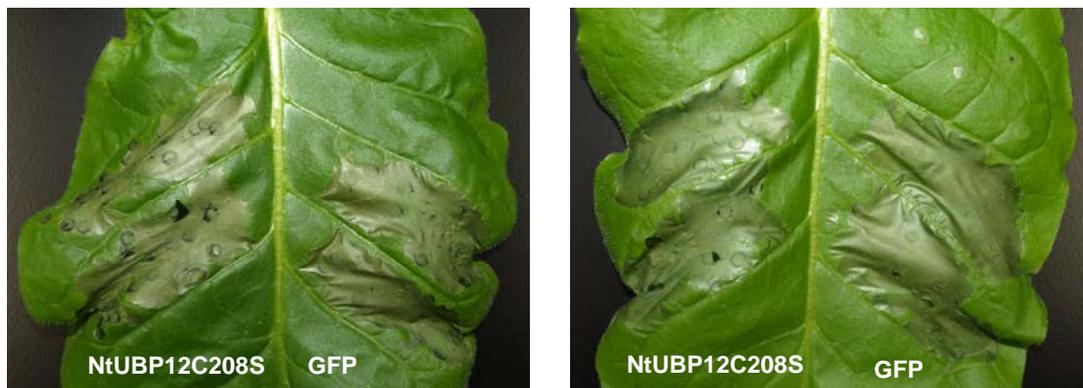
(A) 20 fold Avr9　　　　　　(B) 30 fold Avr9

Figure 3.12 Transient over expression of the noncatalytic mutant NtUBP12 C208S in Cf-9 tobacco fail to suppresses Avr9 elicited HR.

HR development in Cf-9 tobacco leaf segments over expressing GFP-NtUBP12 (left segments) and GFP (right segments) following Avr9 elicitor infiltration. Picture taken after 4 days Avr9 elicitor infiltration.

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3.3.3 Analysis of GFP-NtUBP12 localization during transient expression by confocal microscopy

The application of fluorescence proteins as fusion tags allows *in vivo* analysis of many aspects of protein including: trafficking, turnover, interaction and movements (Sparkes, 2006). The subcellular distribution of GFP-NtUBP12 during transient overexpression in *Nicotiana benthamiana* was analysed by laser-scanning confocal microscopy to detect GFP fluorescence (figure 3.13).

Fusion protein localisation was analysed during the observed peak of transient expression at two days after agroinoculation of GFP-NtUBP12Wt, GFP-NtUBP12C208S and control GFP constructs. Confocal analysis detected GFP-NtUBP12C208S fluorescence in nucleus, but failed to detect GFP-NtUBP12Wt. It might be reflected in a correspondingly low GFP signal strength to this construct. Despite the weak transgene expression of NtUBP12Wt, GFP fluorescence was clearly visible in both nuclear and cytoplasmic compartments during transient over expression of GFP-NtUBP12Wt and GFP-NtUBP12C208S.

(A) GFP-NtUBP12 C208S   (B) GFP
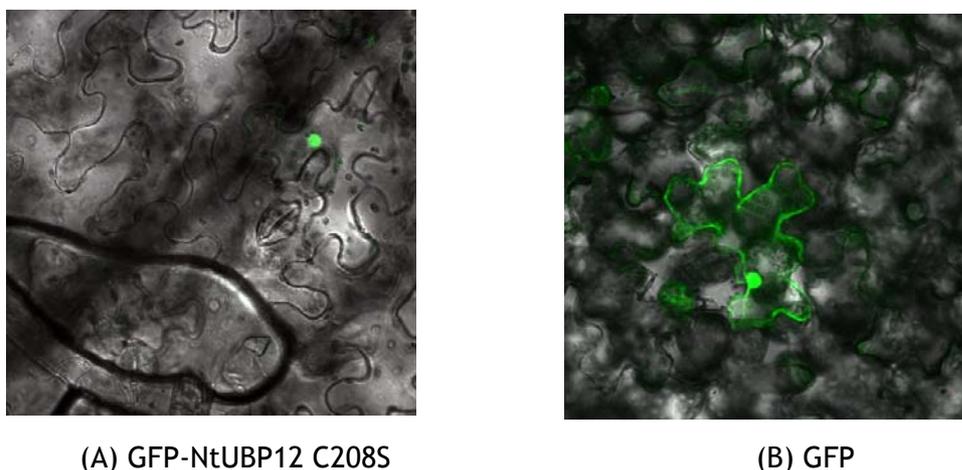
Figure 3.13 The subcellular districution of GFP-NtUBP12 during transient overexpression by confocal microscopy.(A) GFP-NtUBP12 C208S (B) GFP.

3.4 Discussion

3.4.1 *AtUBP12* and *AtUBP13* silencing

Based on the reported transcriptional supression of a solanaceous UBP gene during HR in *N. benthamiana* (Kim et al., 2006), experiments were conducted to investigate possible involvement of the orthologous *Arabidopsis* UBP enzymes AtUBP12 and AtUBP13 in disease resistance. A prior analysis of segmental chromosome duplication indicated that *AtUBP12* and *AtUBP13* arose from a gene duplication event and their recent evolutionary divergence was confirmed in a phylogenetic analysis of the *Arabidopsis* UBP enzymes (Ewan, 2008).

The detection of altered downstream signalling or pertubations in pathogen resistance in single gene mutants of *AtUBP12* and *AtUBP13* may be obscured by functional redundancy between the two genes. Functional redundancy typically results from incomplete specification between duplicated genes (Pickett and Meeks-Wagner, 1995) and has been previously reported between an *Arabidopsis* UBPs *AtUBP15* and *AtUBP16* which regulate cell proliferation and leaf development (Liu et al., 2008). In this study, potential functional redundandancy between *AtUBP12* and *AtUBP13* was investigated using approaches based on transgenic RNAi silencing.

RT PCR result showed that transgenic expression of *AtUBP13* hpRNA fragment to induce co-silencing of *AtUBP13* and *AtUBP12* indicated a functional overlap between these genes.

RT-PCR analysis of different T_2 generation UBP_RNAi lines demonstrated marked variability in the extent of co-silencing. Despite co-silencing levels of *AtUBP12* and *AtUBP13*, there were only two lines showing silencing (line #23 and #26) while all 26 lines showed silencing in T_1. This observation suggests that T_1 co-silencing efficiency of AtUBP12 and AtUBP13 was potentially higher than that seen in the T_2 lines. Surprisingly, RT-PCR in the T_3 generation from two lines were observed only 1 line (line#23) showing clear reduction of *AtUBP12* and *AtUBP13* in mRNA levels. Added to the marked reduction in silencing efficiency in T3, line #26 expression levels was effectively the same

as wildtype and suggested that the expression of the transgene was affected in later T$_3$ generation. This result indicates either AtUBP12 or AtUBP13 are essential for normal plant development. Supporting above mentioned results Ewan (2008) found that double knock out of AtUBP12 and AtUBP13 is embryo lethal, indicating that this gene AtUBP12 or AtUBP13 needed for normal plant development.

The growth retarded phenotype of silenced line *AtUBP12* and *AtUBP13* under short day growth conditions also indicates the requirement of either *AtUBP12* or *AtUBP13* for normal plant development. Ewan (2008) found that *AtUBP12* knock out (KO) plants showed early flowering under short day growth conditions. This result could suggest that *AtUBP12* may function as a regulator of photoperiod perception.

These findings were confirmed in a recent screen for *Arabidopsis* developmental phenotypes in a collection of 39 mutant lines corresponding to 25 of the 27 UBP genes (Liu et al., 2008). Mutant *ubp12* lines were not analysed by Liu *et al* (Liu et al., 2008), while mutant of *ubp13* were analysed but there is no detectable phenotypes.

The potential involvement of *AtUBP12* and *AtUBP13* in disease resistance was investigated by using the *Pseudomonas* virulent DC3000 line. Characterisation of *AtUBP12* and *AUBP13* silencing during infection with virulent *Pseudomonas* DC3000 showed alteration in the disease resistance. The pathogen number was reduced in the silenced lines. This result shows that *AtUBP12* and *AUBP13* is a negative regulator of defence response.

3.4.2 Overexpression of Nt-UBP12Wt supresses the Cf-9 triggered HR in tobacco

The full length cDNA sequence was established by Richard A. Ewan, in this study I have successfully generated GFP tagged NtUBP12.

Results presented in this chapter confirm that solanaceous UBP12 proteins function as a negative regulator (or regulators) of the Cf-9 triggered HR. Transient overexpression of NtUBP12Wt proteins in tobacco demonstrates a gain of function HR supression phenotype.

The result from figure 3.12 indicates that overexpression of the NtUBP12 C208S active site mutant is unable to suppress cell death where as wild type protein is able to suppress cell death. This result confirming that increased *in vivo* UBP12 activity is the specific cause of cell death suppression.

CHAPTER 4

Isolation and Characterization of DeUbiquitin Specific Protease 12 Interactor 1 (DIN1)

4.1 Introduction

The results presented in chapter 3 strongly indicate that DeUbiquitin Specific Protease 12 (UBP12) and DeUbiquitin Specific Protease 13 (UBP13) play a critical role in defence against the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* (virulent *Pst* DC3000). Therefore, proteins that interact with UBP12 or UBP13 are also likely to play an important role in plant defence. To identify interactors of UBP12 we performed a yeast two hybrid (Y2H) screen using an *Arabidopsis* 7 days old seedling cDNA Y2H library and UBP12 as the bait.

The Y2H system was originally developed by Fields and Song (1989) as a genetic assay to detect protein-protein interactions in a cellular setting. This screen was performed in bakers yeast *Saccharomyces cerevisiae* (Gietz et al., 1997). In Fields and Song experiment, a yeast protein SNF1 protein (serine-threonine-specific kinase) is fused to the binding domain (BD) and another yeast protein SNF4 is fused to the activation domain (AD) of the same transcription activator (in this study GAL4). When both the hybrids are present in a single cell, the interaction between SNF1 and SNF4 allows AD to come in the vicinity of BD constituting an active transcription factor, which can easily be assayed by expression of some downstream reporter gene (Fields and Song, 1989) (Mukherjee et al., 2001). Based on this initial experiment, the cDNA of a protein of interest is cloned in an appropriate plasmid vector, which will express it as a BD fusion protein in Y2H screening; this BD fusion protein is known as the bait. Similarly a prey is prepared by cloning the cDNA of another protein of interest in a plasmid vector, which will express it as an AD fusion protein in yeast cells (Mukherjee et al., 2001). Then both the plasmids are co-transformed into yeast cells and growth on selective medium.

Positive transformants were tested for β-galactosidase activity (one of the reporters). Plasmids DNA was isolated from the positive clones and assayed in a bait dependency test using mating strategy (Kolonin et al., 2000). Only clones that showed β-galactosidase activity when co-expressed with the UBP12 bait but not when co-expressed with the control bait was considered to be bait dependent interactors. The identity of bait dependent interactor was determined by sequencing the plasmid DNA.

Using the Arabidopsis cDNA Y2H library, we screened 2.3×10^7 clones against the UBP12 bait. Screening 2.3×10^7 transformant yielded 1 strong putative UBP12 dependent interactor, which produced high levels of β-galactosidase activity when co-expressed with the BD-UBP12 but not when co-expressed with the control. This clone was considered to be <u>De</u>Ubiquitin Specific Protease 12 <u>In</u>teractor <u>1</u> (DIN1) and was analyzed further.

In order to establish the potential involvement of *DIN1* in plant defence, we will utilised the model plant pathogen system *Arabidopsis* in a loss of function assay. T-DNA of *Agrobacterium tumefacians* were used as mutagens to create loss of function mutations in *Arabidopsis* (Radhamony et al., 2005). The T-DNA insertion lines of *DIN1* were obtained from the GABI-Kat T-DNA collection (Cologne, Germany).

This chapter reports the data for: (i) the isolation and sequence identity of the UBP12 gene's strongest interactor (DIN1); (ii) isolation of *DIN1* T-DNA insertion lines from *Arabidopsis*; (iii) the generation of a N-terminal GFP fused DIN1 for subcellular localization studies.

4.2 Identification of DIN1 as an interactor of UBP12 in yeast two hybrid system

A Y2H screen was undertaken to identify proteins that may interact with pLexA-UBP12. The full length 3376 bp of *UBP12* cDNA that was used as "bait construct" was kindly supplied by Richard Ewan (Ph.D student in Sadanandom group). A seven day-old light-grown *Arabidopsis* seedling cDNA library was used for the Y2H screening. The Y2H screen was carried out by Dualsystems Biotech AG, Zurich, Switzerland. 4 independent putative interactor clones were obtained after screening 2.3×10^7 yeast clones. Only one of these 4 independent clones, produced high levels of β-galactosidase activity when co-expressed with the BD-UBP12 but not when co-expressed with the control (figure 4.1A). This clone was considered to be DIN1 and was analyzed further.

The insert DNA sequence of the *DIN1* clone was obtained from Dualsystems Biotech AG and found to be 656 bp long. Database search using the obtained *DIN1* sequence revealed that *DIN1* nucleotide sequence matched a sequence of a genomic region located in chromosome 3 in *Arabidopsis* (ID number: AT3G53900).

The complete cDNA sequence of AT3G53900 was obtained and aligned against *DIN1* nucleotide sequence. As shown in figure 4.1B, this analysis revealed that *DIN1* nucleotide sequence was identical with AT3G53900 cDNA. DIN1 has a role in coding uracyl phosphoribosyltransferase that forms uridine 5'monophosphate (UMP) from uracil and phosphoribosyl-α-pyrophosphate (PRPP) (Andersen et al., 1992; Islam et al., 2007). There are at least six *UPRT* genes in the *Arabidopsis* genome (Kafer et al., 2004) and DIN1 found to be *UPRT3*.

(A)

pLexA-UBP12	pLexA-p53	pLexA-laminC	
UBP12_prey_01.1			
UBP12_prey_04.1			
UBP12_prey_04.2 (DIN1)			
pACT2_largeT			

(B)

```
            1        10        20        30        40        50        60        70        80        90        100       110       120       130
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+|
cDNA_AT3G53900  AAAATCTCGAACCAGATTCGGACATCTCGTCGAAAATGGCGTGCTCAATTGGTAACGCTTTCAGGTGTTCTTCTGATACGCTGCGTTTTGCTCCCCGACAACAATGCAGCAGCCGCCTAAACCCTAATCC
DIN1_pLexA                                       ATGGCGTGCTCAATTGGTAACGCTTTCAGGTGTTCTTCTGATACGCTGCGTTTTGCTCCCCGACAACAATGCAGCAGCCGCCTAAACCCTAATCC
Consensus       ...............................ATGGCGTGCTCAATTGGTAACGCTTTCAGGTGTTCTTCTGATACGCTGCGTTTTGCTCCCCGACAACAATGCAGCAGCCGCCTAAACCCTAATCC

            131      140       150       160       170       180       190       200       210       220       230       240       250       260
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+|
cDNA_AT3G53900  TTCCTCTTTCCTCTCCTTTAATTCCTCCCCAATTCTCGCACAGAATTTGGGTGCTTCTTCTTCGTCACTTTCCCGACGGACTATACGAGCTAGAACAAAAATGGCTGCGTCGGAGGCTTCCATTAACGGG
DIN1_pLexA      TTCCTCTTTCCTCTCCTTTAATTCCTCCCCAATTCTCGCACAGAATTTGGGTGCTTCTTCTTCGTCACTTTCCCGACGGACTATACGAGCTAGAACAAAAATGGCTGCGTCGGAGGCTTCCATTAACGGG
Consensus       TTCCTCTTTCCTCTCCTTTAATTCCTCCCCAATTCTCGCACAGAATTTGGGTGCTTCTTCTTCGTCACTTTCCCGACGGACTATACGAGCTAGAACAAAAATGGCTGCGTCGGAGGCTTCCATTAACGGG

            261      270       280       290       300       310       320       330       340       350       360       370       380       390
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+|
cDNA_AT3G53900  AGCAACAGGATGCTGGTCTTTGTGCCACCACATCCTTTGATCAAACACTGGATCTCTGTTCTGAGAAATGAACAAACTCCTTGTCCTGTTTTCAGGAATGCAATAGCTGAACTAGGGAGGATTACTCATGT
DIN1_pLexA      AGCAACAGGATGCTGGTCTTTGTGCCACCACATCCTTTGATCAAACACTGGATCTCTGTTCTGAGAAATGAACAAACTCCTTGTCCTGTTTTCAGGAATGCAATAGCTGAACTAGGGGAGATTACTCATGT
Consensus       AGCAACAGGATGCTGGTCTTTGTGCCACCACATCCTTTGATCAAACACTGGATCTCTGTTCTGAGAAATGAACAAACTCCTTGTCCTGTTTTCAGGAATGCAATAGCTGAACTAGGGAGGATTACTCATGT

            391      400       410       420       430       440       450       460       470       480       490       500       510       520
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+|
cDNA_AT3G53900  ATGAAGCATCACGAGAGTGGCTGCCAACTGTTGTGGGTGAAATAATGTCTCCAATGGGTCCTGCTTCTGTTGAATTCATTGATCCTAGAGAGCCTATTGCGGTTGTTCCGATATTAAGAGCTGGTCTTGC
DIN1_pLexA      ATGAAGCATCACGAGAGTGGCTGCCAACTGTTGTGGGTGAAATAATGTCTCCAATGGGTCCTGCTTCTGTTGAATTCATTGATCCTAGAGAGCCTATTGCGGTTGTTCCGATATTAAGAGCTGGTCTTGC
Consensus       ATGAAGCATCACGAGAGTGGCTGCCAACTGTTGTGGGTGAAATAATGTCTCCAATGGGTCCTGCTTCTGTTGAATTCATTGATCCTAGAGAGCCTATTGCGGTTGTTCCGATATTAAGAGCTGGTCTTGC

            521      530       540       550       560       570       580       590       600       610       620       630       640       650
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+|
cDNA_AT3G53900  TCTTGCGGAACACGCATCCTCAGTTTTACCTGCTAATAAAATTTATCATTTAGGAGTAAGTAGAGATGAGAAAACACTTTTACCATCGGTGTATCTTAACAAGTTGCCTGATGAATTCCCAAGAATTCA
DIN1_pLexA      TCTTGCGGAACACGCATCCTCAGTTTTACCTGCTAATAAAATTTATCATTTAGGAGTAAGTAGAGATGAGAAAACACTTTTACCATCGGTGTATCTTAACAAGTTGCCTGATGAATTCCCAAGAATTCA
Consensus       TCTTGCGGAACACGCATCCTCAGTTTTACCTGCTAATAAAATTTATCATTTAGGAGTAAGTAGAGATGAGAAAACACTTTTACCATCGGTGTATCTTAACAAGTTGCCTGATGAATTCCCAAGAATTCA

            651      660       670       680       690       700       710       720       730       740       750       760       770       780
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+|
cDNA_AT3G53900  AGAGTATTTTTGGTCGACCCAGTGCTTGCCACAGGTGGTACCATAATGGCGGCGATGGATCTATTGAAGGAACGTGGTTTGTCTGTTCAGCAAATCAAAGTGATTTGTGCGATTGCTGCACCTCCTGCAC
DIN1_pLexA      AGAGTATTTTTGGTCGACCCAGTGCTTGCCACAGGTGGTACCATAATGGCGGCGATGGATCTATTGAAGGAACGTGGTTTGTCTGTTCAGCAAATCAAAGTGATTTGTGCGATTGCTGCACCTCCTGCAC
Consensus       AGAGTATTTTTGGTCGACCCAGTGCTTGCCACAGGTGGTACCATAATGGCGGCGATGGATCTATTGAAGGAACGTGGTTTGTCTGTTCAGCAAATCAAAGTGATTTGTGCGATTGCTGCACCTCCTGCAC

            781      790       800       810       820       830       840       850       860       870       880       890       900       910
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+|
cDNA_AT3G53900  TATCGAAGCTTAACGAGAATTCCCCGGGCTTCATGTATATGCTGGAATTATTGATCCTGAAGTCAATGAAAAGGGGTACATAATCCCTGGGCTTGGAGACGCTGGAGACCGCAGTTTCGGGACAGAAAC
DIN1_pLexA      TATCGAAGCTTAACGAGAATTCCCCGGGCTTCATGTATATGCTGGAATTATTGATCCTGAAGTCAATGAAAAGGGGTACATAATCCCTGGGCTTnGAGACGCTGGAnACCGCAGTTTCGGGACAGAAAC
Consensus       TATCGAAGCTTAACGAGAATTCCCCGGGCTTCATGTATATGCTGGAATTATTGATCCTGAAGTCAATGAAAAGGGGTACATAATCCCTGGGCTTnGAGACGCTGGAnACCGCAGTTTCGGGACAGAAAC

            911      920       930       940       950       960       970       980       990       1000      1010      1020      1030      1040
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+|
cDNA_AT3G53900  ACATTGGGTGAAGTGATACTTGTATGAGTTGTTAAACTTTAACAGTCACAATCGTTAGATTATATATTGTGTCTCTTGTACTTTGTTTTGATGTGTGATTTCAGGTTCTTGAGAGGGTTTCGATAAAGA
DIN1_pLexA      ACATTGGGTGAAGTGATACTTGTATGAGTTGTTAAACTTTAACAGTCACAATCGTTAGATTATATATTGTGTCTCTTGTACTTTGTTTT-GATGTGTGATTTCA
Consensus       ACATTGGGTGAAGTGATACTTGTATGAGTTGTTAAACTTTAACAGTCACAATCGTTAGATTATATATTGTGTCTCTTGTACTTTGTTTT.GATGTGTGATTTCA...........................

            1041 1050     1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+|
cDNA_AT3G53900  GTCAAAATTTCACAAACAAGAAAAACTGAATTCCTCATTATTACAAAACAGAGAACAAACTTGTCAACCATTTTATGTGTATCATTCTCAATTGTCTACCACAAACCATTTTGGGAAAAAGTATGCAAGA
DIN1_pLexA
Consensus       ...............................................................................................................................

            1171 11801184
            |--------+---+|
cDNA_AT3G53900  AAATCTGAAAAATT
DIN1_pLexA
Consensus       ..............
```

Figure 4.1 Identification DIN1 as an interactor of UBP12 in Y2H system

(A) UBP12 dependancy assay

Representative yeast colonies were transformed with the indicated bait and prey; blue color indicate bait and prey interaction, white colonies indicate no interaction between bait and prey. As prey controls, pLexA-p53 and pLexA-laminC were used. pACT2_largeT was used as bait control, pACT2_largeT expressing the SV40 large T-antigen work as positive control only with pLexA-p53 expressing the murine p53 protein.

(B) Nucleotide sequence alignment of *DIN1* with AT3G53900 cDNA

The *DIN1* sequence obtained from the sequencing facility was aligned against full length cDNA of AT3G53900 obtained from the TAIR website. Alignment colour indicates high conservation (red), low conservation (blue), no conservation (black).

4.3 Isolation of *DIN1* T-DNA Insertion lines

The availability of large populations of T-DNA transformed *Arabidopsis* lines enables reverse genetic analysis of most genes. *Arabidopsis* T-DNA insertion lines corresponding to the *DIN1* gene were obtained from GABI-Kat T-DNA collection (Cologne, Germany).

For genotyping purposes, gene specific primers were designed to flank the genomic T-DNA insertion site using protocols specified by GABI-Kat. To confirm the presence of T-DNA insertions, genomic DNA were PCR amplified using gene specific primer, *DIN1* (corresponding to the 3'-end of the gene) in combination with a T-DNA left border primer (SALK_Lba1). Zygosities of *DIN1* T-DNA insertion lines were confirmed using *DIN1* primers and PCR conditions that prevented product amplification in T-DNA insertion lines.

Based on the PCR result (Figure 4.2), T-DNA was found to be inserted within the *DIN1* gene in plant in lines 1, 2 and 4 but still contain a wild type copy of the *DIN1* gene without a T-DNA insertion indicating that these lines are heterozygotes. The seeds from these 3 plant lines can be used to screen for *DIN1* knock out homozygous lines and investigate the role of *DIN1* in plant defence in the following generations.

(A)

(B)

i

ii

iii
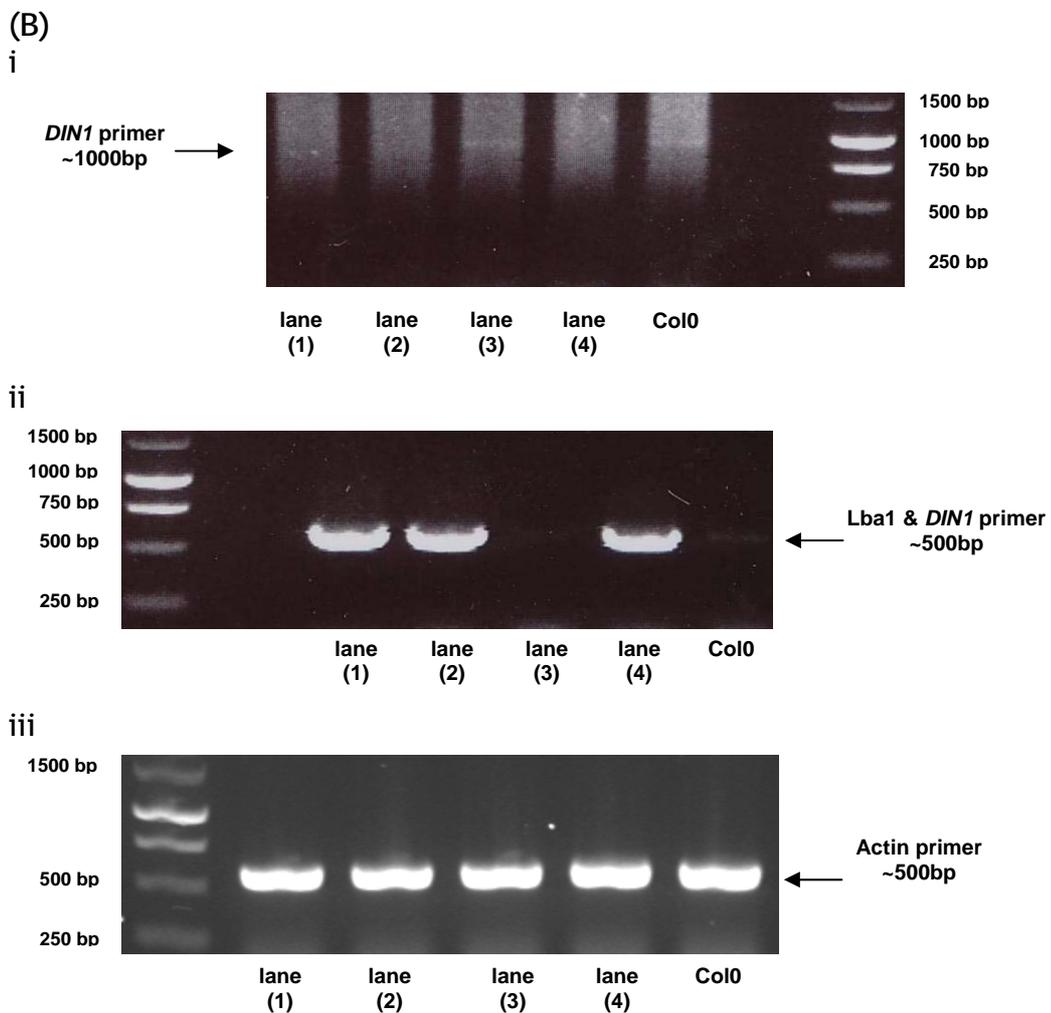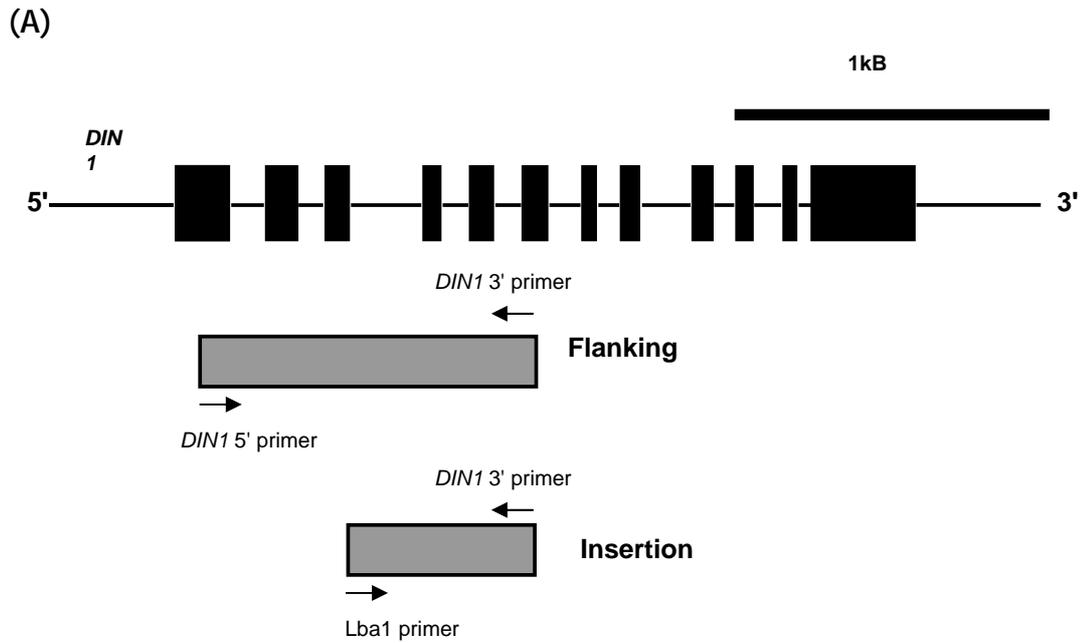
Figure 4.2 PCR analyses of *DIN1* T-DNA insertion lines
(A). Schematic representation of PCR position and size of amplified product by *DIN1* primers and Lba1 primer. PCR amplicon are represented as grey boxes. (B)   (i) Agarose gel showed band produced by *DIN1* primers (flanking); (ii) Agarose gel showed an amplification of Lba1 primers with DIN1 3' primer (insertion); (iii) Agarose gel showed an amplification of actin primer, as indicated by arrow.

The region of DIN1 gene containing the T-DNA insertion was cloned (figure 4.2Bii) into p-GEM®T easy vector and sequenced to ascertain the precise location of the T-DNA insertion. In order to verify that *DIN1* successfully cloned into p-GEM®T easy vector, restriction analysis using *Not*I enzyme was performed and the result are shown in figure 4.3.
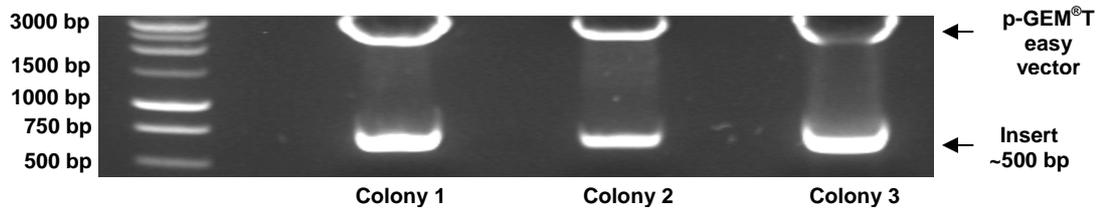
Figure 4.3 Agarose gel analysis showing restriction of *DIN1*-p-GEM®T easy vector using *Not*I enzyme.

Two bands corresponding to 3 kb and 500 bp respectively in colonies 1, 2 and 3 (figure 4.3) indicates that *DIN1* cDNA has been successfully inserted into the p-GEM®T easy vector in these 3 *E.coli* colonies. The corresponding DNA from colony 1 was sent for sequencing by the functional genomics facility at University of Glasgow. The sequence obtained was aligned against the genomic and cDNA sequence obtained from the database available in TAIR website. The sequencing results confirmed the presence of *DIN1* T-DNA insertion within the fifth and sixth exons of the *DIN1* gene (Figure 4.4A and B).

```
                        1         10        20        30        40        50        60        70        80        90        100       110       120       130
                        |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    AAAATCTCGAACCAGATTCGGACATCTCGTCGAAAATGGCGTGCTCAATTGGTAACGCTTTCAGGTGTTCTTCTGATACGCTGCGTTTTGCTCCCCGACAACAATGCAGCAGCCGCCTAAACCCTAATCC
      cDNA_AT3G53900    AAAATCTCGAACCAGATTCGGACATCTCGTCGAAAATGGCGTGCTCAATTGGTAACGCTTTCAGGTGTTCTTCTGATACGCTGCGTTTTGCTCCCCGACAACAATGCAGCAGCCGCCTAAACCCTAATCC
  DIN1_TDNA_insertion
           Consensus    aaaatctcgaaccagattcggacatctcgtcgaaaatggcgtgctcaattggtaacgctttcaggtgttcttctgatacgctgcgttttgctccccgacaacaatgcagcagccgcctaaaccctaatcc

                        131       140       150       160       170       180       190       200       210       220       230       240       250       260
                        |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    TTCCTCTTTCCTCTCCTTTAATTCCTCCCCAATTCTCGCACAGGTATCGAGTATATATATATATTATTCTAATGTCCAAAGCACACTTTTCCAGTAACGCCTTTTGCATATTCAAACTGAATTTTGATT
      cDNA_AT3G53900    TTCCTCTTTCCTCTCCTTTAATTCCTCCCCAATTCTCGCACAG--------------------------------------------------------------------------------------
  DIN1_TDNA_insertion
           Consensus    ttcctctttcctctcctttaattcctccccaattctcgcacag....................................................................................

                        261       270       280       290       300       310       320       330       340       350       360       370       380       390
                        |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    TTCGAATTTCTGGATTATTTTAAAGAATTTGGGTGCTTCTTCTTCGTCACTTTCCCGACGGACTATACGAGCTAGAACAAAAATGGCTGCGTCGGAGGCTTCCATTAACGGGAGCAACAGGATGCTGGTA
      cDNA_AT3G53900    ----------------------------AATTTGGGTGCTTCTTCTTCGTCACTTTCCCGACGGACTATACGAGCTAGAACAAAAATGGCTGCGTCGGAGGCTTCCATTAACGGGAGCAACAGGATGCTGGT-
  DIN1_TDNA_insertion
           Consensus    ........................aatttgggtgcttcttcttcgtcactttcccgacggactatacgagctagaacaaaaatggctgcgtcggaggcttccattaacgggagcaacaggatgctggt.

                        391       400       410       420       430       440       450       460       470       480       490       500       510       520
                        |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    ATAAAACTCTAGGATTTCAATTTTCAGATTTTTTTTGAGTTTGATTTGAAAGTGAAAGATGTGAATTTGATTCTGGTGTTTCAGGTCTTTGTGCCACCACATCCTTTGATCAAACACTGGATCTCTGTTC
      cDNA_AT3G53900    --------------------------------------------------------------------------------CTTTGTGCCACCACATCCTTTGATCAAACACTGGATCTCTGTTC
  DIN1_TDNA_insertion
           Consensus    ................................................................................ctttgtgccaccacatcctttgatcaaacactggatctctgttc

                        521       530       540       550       560       570       580       590       600       610       620       630       640       650
                        |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    TGAGAAATGAACAAACTCCTTGTCCTGTTTTCAGTGAGTAGATAAGATTCTTATGAGTATTTCTGCGATTTAACCAGTGTTTGTTGTTAAAGAACTGAATTTTACATCCATTTTTTTTTTTTTTTTTTTTT
      cDNA_AT3G53900    TGAGAAATGAACAAACTCCTTGTCCTGTTTTCAG---------------------------------------------------------------------------------------------
  DIN1_TDNA_insertion
           Consensus    tgagaaatgaacaaactccttgtcctgttttcag..........................................................................................

                        651       660       670       680       690       700       710       720       730       740       750       760       770       780
                        |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    GATTCGTTGGACAATCTTTAGCTCTTAGATTGGTGTTATCTGCTACATTGCGTTTGAATATGAGTTTTTTTTAGGTTTGCTACTAGTATARAGTCTGTGTTGATCTTTAAAGCTTGAGTTTTTTTTAATTATC
      cDNA_AT3G53900    --------------------------------------------------------------------------------------------------------------------------
  DIN1_TDNA_insertion
           Consensus    ..........................................................................................................................

                        781       790       800       810       820       830       840       850       860       870       880       890       900       910
                        |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    TAGGGAATGCAATAGCTGAACTAGGGAGATTACTCATGTATGAAGCATCACGAGAGTGGCTGGTGCGTAGAATTTCACATCTCTATTATTCTTGAATTCTTCCTACCCGTTTTTTGCTTATTGTTTTTTG
      cDNA_AT3G53900    ----GAATGCAATAGCTGAACTAGGGAGATTACTCATGTATGAAGCATCACGAGAGTGGCTG--------------------------------------------------------------
  DIN1_TDNA_insertion
           Consensus    ....gaatgcaatagctgaactagggagattactcatgtatgaagcatcacgagagtggctg.................................................................

                        911       920       930       940       950       960       970       980       990       1000      1010      1020      1030      1040
                        |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    TTTTTTGCTCTATGCGGTTAAGCCAACTGTTGTGGGTGAAATAATGTCTCCAATGGGTCCTGCTTCTGTTGAATTCATTGATCCTAGAGAGCCTATTGCGGTTTGTTTATCCCTCAAACTCTTCCTGTCT
      cDNA_AT3G53900    ----------------------CCAACTGTTGTGGGTGAAATAATGTCTCCAATGGGTCCTGCTTCTGTTGAATTCATTGATCCTAGAGAGCCTATTGCGGTT--------------------
  DIN1_TDNA_insertion                          CCAACTGTTGTGGGTGAAATAATGTCTCCAATGGGTCCTGCTTCTGTTGAATTCATTGATCCTAGAGAGCCTATTGCGGTTTGTTTATCCCTCAAACTCTTCCTGTCT
           Consensus    ......................CCAACTGTTGTGGGTGAAATAATGTCTCCAATGGGTCCTGCTTCTGTTGAATTCATTGATCCTAGAGAGCCTATTGCGGTTtgtttatccctcaaactcttcctgtct

                        1041 1050      1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170
                        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    ATATTTTCTTTTCGACCTTACATGTAAGCGTTTCTAATTCTTGGTTTTGTCTCTGTGTAGGTTGTTCCGATATTAAGAGCTGGTCTTCGTCTTGCGGAACACGCATCCTCAGTTTTACCTGCTAATAAAA
      cDNA_AT3G53900    ----------------------------------------------------------------GTTCCGATATTAAGAGCTGGTCTTCGTCTTGCGGAACACGCATCCTCAGTTTTACCTGCTAATAAAA
  DIN1_TDNA_insertion    ATATTTTCTTTTCGACCTTACATGTAAGCGTTTCTAATTCTTGGTTTTGTCTCTGTGTAGGTTGTTCCGATATTAAGAGCTGGTCTTCGTCTTGCGGAACACGCATC
           Consensus    atattttcttttcgaccttacatgtaagcgtttctaattcttggttttgtctctgtgtaggttGTTCCGATATTAAGAGCTGGTCTTCGTCTTGCGGAACACGCATCctcagttttacctgctaataaaa

                        1171 1180      1190      1200      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
                        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    TTTATCATTTAGGTTAGTAATCTAGCTATATATCATCTCTTGCCTGAACTTCGGATGTCAAAAATGAAAACTTTTATCAAATAGCTTATTGATGTCCCCGTGCTTCTTTTCTTTTACAGGAGTAAGTAAGA
      cDNA_AT3G53900    TTTATCATTTAGG------------------------------------------------------------------------------------------------------------AGTAAGTAAGA
  DIN1_TDNA_insertion
           Consensus    tttatcatttagg..................................................................................................agtaagtaaga

                        1301      1310      1320      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430
                        |---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    GATGAGAAAACACTTTTACCATCGGTGTATCTTAACAAGTAAGAACTTCTCTGCAGCATTGTGTTTGTAGTTACTGAAGTGTTTATAATAACACCTTTGATACATCTTCTTTAGGTTGCCTGATGAATTT
      cDNA_AT3G53900    GATGAGAAAACACTTTTACCATCGGTGTATCTTAACAAGT----------------------------------------------------------------------------TGCCTGATGAATTT
  DIN1_TDNA_insertion
           Consensus    gatgagaaaacacttttaccatcggtgtatcttaacaagt............................................................................tgcctgatgaattt

                        1431 1440      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560
                        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    CCCAAGAATTCAAGAGTATTTTTGGTCGACCCAGTGCTTGCCACAGGTAAGAAAGTCTTATCATTTTAGCCACCTCCATATTAGAAGTATAGTTAGGGTTTGATGTTCAAATGTACACGATAATATGTGA
      cDNA_AT3G53900    CCCAAGAATTCAAGAGTATTTTTGGTCGACCCAGTGCTTGCCACAGGT-----------------------------------------------------------------------------
  DIN1_TDNA_insertion
           Consensus    cccaagaattcaagagtattttggtcgacccagtgcttgccacaggt..............................................................................

                        1561 1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680      1690
                        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    ATGTGAACGCATGACATGGTAATACGAATGTTTCATAGCTAGAAGCGTAGGTTTAGTAACTTAGTAACTGTATTTTAGGTGGTACCATAATGGCGGCGATGGATCTATTGAAGGAACGTGGTTTGTCTG
      cDNA_AT3G53900    ------------------------------------------------------------------------GGTACCATAATGGCGGCGATGGATCTATTGAAGGAACGTGGTTTGTCTG
  DIN1_TDNA_insertion
           Consensus    ........................................................................ggtaccataatggcggcgatggatctattgaaggaacgtggtttgtctg

                        1691 1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800      1810      1820
                        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    TTCAGCAAATCAAAGTGGTAAGCATATATATAGGAAACAGCTTTTGTCAGAAATCGAAGTGTTAATTGTTTTGATTTTTCTGATTACAGAATTTGTGCGATTGCTGCACCTCCTGCACTATCGAAGCTTAA
      cDNA_AT3G53900    TTCAGCAAATCAAAGTG-----------------------------------------------------------------ATTTGTGCGATTGCTGCACCTCCTGCACTATCGAAGCTTAA
  DIN1_TDNA_insertion
           Consensus    ttcagcaaatcaaagtg.................................................................atttgtgcgattgctgcacctcctgcactatcgaagcttaa

                        1821 1830      1840      1850      1860      1870      1880      1890      1900      1910      1920      1930      1940      1950
                        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    CGAGAAATTCCCCGGGTAATCAATAGCAAAAGATCAACTCTCACCTTGCTTACCATTTAGTTAACTCTCTTTTTACTAAACTGTTTGGCTACTATTTGAAACATTTCAGGCTTCATGTATATGCTGGAAT
      cDNA_AT3G53900    CGAGAAATTCCCCGGG----------------------------------------------------------------------------------------CTTCATGTATATGCTGGAAT
  DIN1_TDNA_insertion
           Consensus    cgagaaattccccggg....................................................................................cttcatgtatatgctggaat

                        1951 1960      1970      1980      1990      2000      2010      2020      2030      2040      2050      2060      2070      2080
                        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    TATTGATCCTGAAGTCAATGAAAAGGGGTAAGTCATTAGGTTCTTTGGCTTTCCATATGAAAACTGGCGATTGTGATTTTAATGTCCTTATGTATTTGTCATGAACTAGGTACATAATCCCTGGGCTTGGA
      cDNA_AT3G53900    TATTGATCCTGAAGTCAATGAAAAGGGGTA--------------------------------------------------------------------------CATAATCCCTGGGCTTGGA
  DIN1_TDNA_insertion
           Consensus    tattgatcctgaagtcaatgaaaaggggta..........................................................................cataatccctgggcttgga

                        2081 2090      2100      2110      2120      2130      2140      2150      2160      2170      2180      2190      2200      2210
                        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    GACGCTGGAGACCGCAGTTTCGGGACAGAAACACATTGGGTGAAGTGATACTTGTATGAGTTGTTAAACTTTAACAGTCACAATCGTTAGATTATATATTGTGTCTCTTGTACTTTGTTTTTGATGTGTG
      cDNA_AT3G53900    GACGCTGGAGACCGCAGTTTCGGGACAGAAACACATTGGGTGAAGTGATACTTGTATGAGTTGTTAAACTTTAACAGTCACAATCGTTAGATTATATATTGTGTCTCTTGTACTTTGTTTTTGATGTGTG
  DIN1_TDNA_insertion
           Consensus    gacgctggagaccgcagtttcgggacagaaacacattgggtgaagtgatacttgtatgagttgttaaactttaacagtcacaatcgttagattatatattgtgtctcttgtactttgtttttgatgtgtg

                        2211 2220      2230      2240      2250      2260      2270      2280      2290      2300      2310      2320      2330      2340
                        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      gDNA_AT3G53900    ATTTCAGGTTCTTGAGAGGGTTTCGATAAGAGTCAAAATTTCACAAACAAGAAAAACTGAATTCCTCATTATTACAAAACAGAGAACAAACTTGTCAACCATTTTATGTGTATCATTCTCAATTGTCTA
      cDNA_AT3G53900    ATTTCAGGTTCTTGAGAGGGTTTCGATAAGAGTCAAAATTTCACAAACAAGAAAAACTGAATTCCTCATTATTACAAAACAGAGAACAAACTTGTCAACCATTTTATGTGTATCATTCTCAATTGTCTA
  DIN1_TDNA_insertion
           Consensus    atttcaggttcttgagagggtttcgataagagtcaaaatttcacaaacaagaaaaactgaattcctcattattacaaaacagagaacaaacttgtcaaccattttatgtgtatcattctcaattgtcta

                        2341 2350      2360      2370      2380 2386
                        |--------+---------+---------+------+-----|
      gDNA_AT3G53900    CCACAAACCATTTTGGGAAAAAGTATGCAAGAAAATCTGAAAAATT
      cDNA_AT3G53900    CCACAAACCATTTTGGGAAAAAGTATGCAAGAAAATCTGAAAAATT
  DIN1_TDNA_insertion
           Consensus    ccacaaaccattttgggaaaaagtatgcaagaaaatctgaaaaatt
```

(B)

Figure 4.4 Sequence analyses of *DIN1* T-DNA insertion lines

(A) *DIN1* sequence obtained from the sequencing facility was aligned against gDNA and cDNA of AT3G53900 obtained from TAIR website.
(B) Schematic representation of *DIN1* genomic DNA structure, complete with 12 exons. Black rectangle boxes and black thin lines represent exons and introns respectively. The ATG mark the position of the start codon. Grey triangle represents the T-DNA insertions.

4.4 Generation of GFP-DIN1 fusion proteins

We wished to investigate the subcellular localization of DIN1. Therefore GFP was fused to the N-terminus of *DIN1* (Palmer and Freeman, 2004).

To generate the N-terminal GFP tagged DIN1 fusion constructs, *DIN1* cDNA was sub cloned into D-TOPO vector. To get the sub clone into D-TOPO vector, first the cDNA was amplified by Polymerase Chain Reaction (PCR) using specific *DIN1* specific primers that have the additional 4 bases in the forward primer (CACC) (figure 4.4A). These CACC bases at the 5' primer allow the amplified PCR product to ligate directionally into the D-TOPO vector. 5 μl of PCR products were analysed on agarose gel (figure 4.4B) to confirm positive PCR amplification. The rest of the PCR product was used in ligation reaction with the D-TOPO vector.

(A)

1 kB

5' UTR   CDS   3' UTR

PCR amplicon

(B)

1000 bp
750 bp
500 bp

Replicates 1   Replicates 2
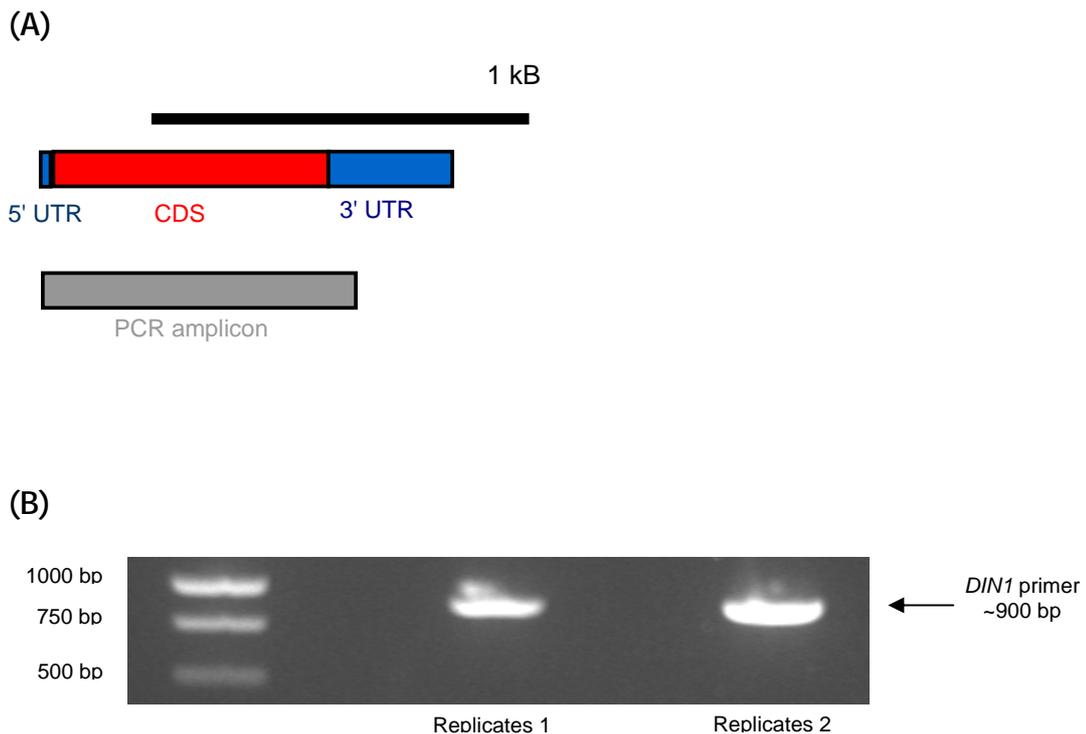
DIN1 primer
~900 bp

Figure 4.5 PCR for preparation of subcloning into D-TOPO vector

(A) Schematic representation of PCR position and size of amplified product by *DIN1* primers. PCR amplicon of *DIN1* are represented as grey boxes.
(B) Agarose gel analysis of PCR showing ampification of gene encoding *DIN1* for preparation of subcloning into D-TOPO vector.

In order to verify that *DIN1* successfully cloned into D-TOPO, restriction analysis was performed. Plasmid DNA of D-TOPO-*DIN1* was extracted and digested with *Not*I enzyme (figure 4.6). The expected size of the DNA fragment is 3480bp, since the size of D-TOPO vector is 2580 bp and *DIN1* is arround 900 bp totally 3480bp. The presence of single band at the 3480bp in colony 1, 2 and 4 indicates that *DIN1* has successfully been cloned into the plasmid within these 3 *E.coli* colonies.

Plasmid DNA of D-TOPO *DIN*1 was sequenced by the functional genomics facility at University of Glasgow. The sequencing result is shown on figure 4.7.
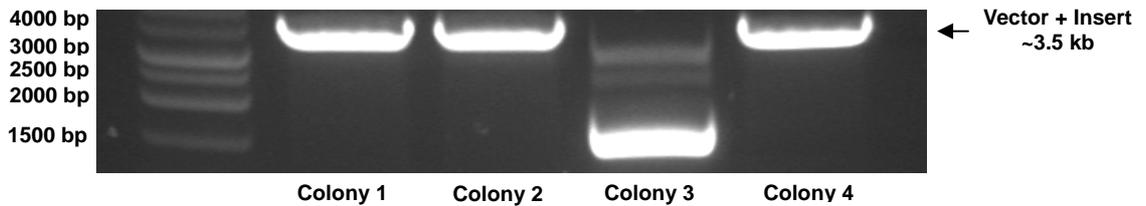
Figure 4.6 Agarose gel analysis showing restriction digestion of *DIN1*-D-TOPO using *Not*I enzyme.
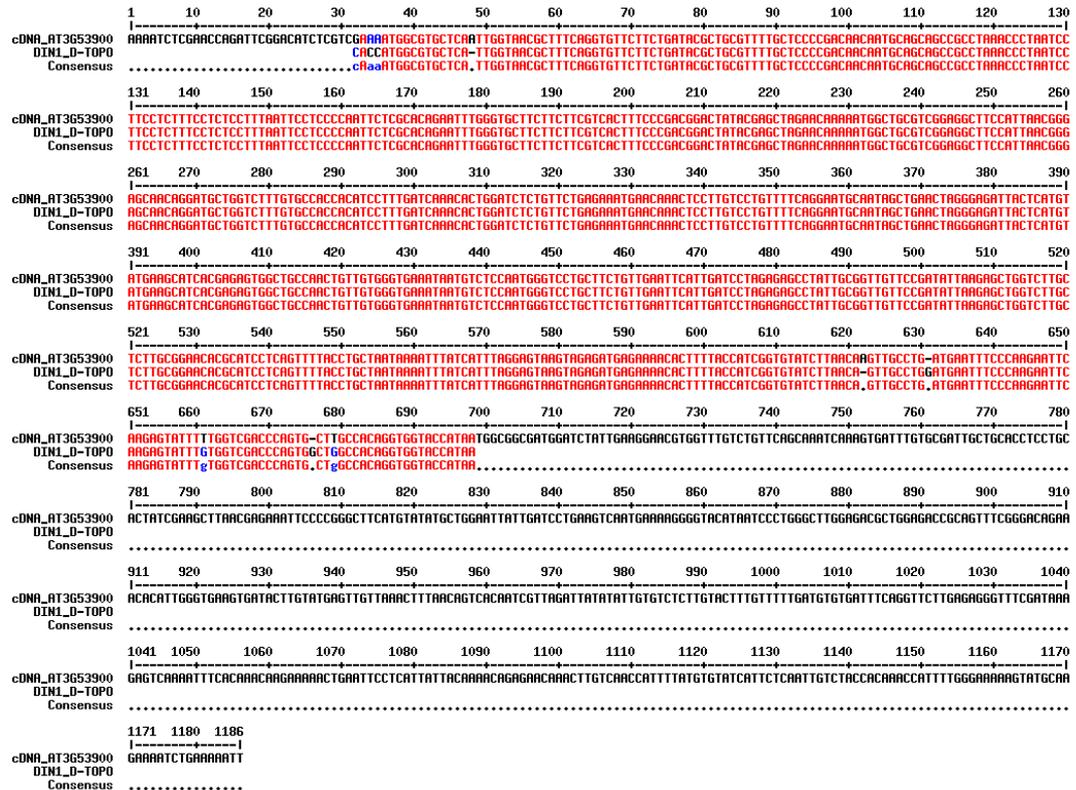
Figure 4.7 Nucleotide sequence alignment of *DIN1*

The *DIN1* sequence obtained from the sequencing facility was aligned against cDNA of *DIN1* obtained from the TAIR website. Alignment colour indicates high conservation (red), low conservation (blue), no conservation (black).

Sequencing data confirms that *DIN*1 was partially cloned into D-TOPO with 4 frame shift mutations. cDNA from the respective clone was transferred into the pGWB6 destination vector by Gateway LR based recombination to generate N-terminal GFP tagged DIN1 fusion constructs. The aim was to introduce GFP-DIN1 fusion construct into *N. benthamiana* by performing an Agrobacterium mediated transient assay to observe subcellular localization. However, due to time constraint of the project, the project was brought to an end at this time.

66

4.5 Discussion

The ability to perceive and initiate a defence response against pathogenic microorganisms is paramount to the success of all plant life. Unlike animals, plant lack a circulating immune system recognizing microbial pathogens (Liu and Coaker, 2008). Plants have evolved intricate defence mechanisms to cope with the wide array of microbial pathogens they encounter (Gray, 2002). The identification of SGT1 and COP9 as essential components for R gene-mediated disease resistance suggest that the ubiquitin plays an important role in plant defence (Gray, 2002; Devoto, et al., 2003). Recently, based on microarray data Kim et al., suggest that UBP6 and UBP12 may play a role in the defence response (Kim et al., 2006). Supporting this finding, the results in chapter 3 indicate that UBP12 play a critical role in defence against bacterial pathogen (virulent *Pst* DC3000). Experiments were conducted to investigate the role of DIN1 interactor of UBP12 in plant defence.

The importance of Uracyl phosphoribosyltransferase in the pyrimidine pathway is well established (Kafer et al., 2004; Islam et al., 2007). Initially, uracil phosphoribosyltransferase activity was reported in *Arabidopsis thaliana* and microorganisms (Martinussen, 1994; Kafer et al., 2007), but recently, Li et al., reported that human UPRTase protein was strongly expressed in blood leukocytes, liver, spleen, and thymus (Li et al., 2007).

Based on the results of a data base search and Kafer et al., (2004), there are at least six *UPRT* genes in the *Arabidopsis* genome. They are *UPRT1* (AT1G55810), *UPRT2* (AT3G27190), *UPRT3* or *DIN1* (AT3G53900), *UPRT4* (AT4G26510), *UPRT5* (AT5G40870) and *UTK* (AT3G27440). Sequence comparison six *UPRT* genes in *Arabidopsis* were shown in figure 4.8.

```
              1        10        20        30        40        50        60        70        80        90       100       110       120       130
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AT5G40870     MPEDSSSLDYAMEKASGPHFSGL-RFDGLLSSSPPNSSVVSSLRSAVSSSSPSSSD-PEAPKQPFIIGVSGGTASGKTTVYCDMIIQQLHDHRVYLVNQDSFYRGLTSEELQRYQEYNFDHPDAFDTEQLL
AT3G27190     MPEDSTAIDYVMEKASGPHFSGL-RLDGLLSS-PSKSSVVSSPSHFRLSNSSFSATDDPAAPHQPFVIGVTGGTASGKTTVYCDMIIQQLHDHRIVLVNQDSFYRGLTSEELEHVQEYNFDHPDAFDTEQLL
AT1G55810      MASKSDVNIIETSSKVHFSGFHQMDGLASNRPEQMAEE------------------EEHGQPFVIGVAGGAASGKTTVYCDMIMQQLHDQRAVVYNQDSFYHNVNEVELVRVHDYNFDHPDAFDTEQLL
AT3G27440                      MEKLSNGVRDHCLISDYVSPSAPAPLKQPFVIGVAGGTASGKTTVYCNMIHSQLHDQRVYLVNQDSFYHSLTKEKLNKVHEYNFDHPDAFNTEVLL
AT4G26510                                                       MIIQQLHDQRVYLINLDSFYHNLTEEELARVHEYNFDHPDAFDTEHLL
AT3G53900
Consensus     ............................................................ni.qqlhd.r.vl.n.dsfy..lt.eel..v.eynfdhpdafdte.ll

              131       140       150       160       170       180       190       200       210       220       230       240       250       260
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AT5G40870     HCAETLKSGQPYQVPIYDFKTHQRRSDTFRQYNASDVIILEGILVFHDSRVRNLMNMKIFYDTDADYRLARRIRRDTYERGRDYNSVLEQYAKFVKPAFDDFVLPSKKYADVIIPRGGDNHVAVDLITQH
AT3G27190     HCVDILKSGQPYQIPIYDFKTHQRKVDAFRQYNACDVIILEGILVFHDSRVRDLMNMKIFYDTDADYRLARRIRRDTYERGRDYDSVLEQYAKFVKPAFDDFVLPSKKYADVIIPRGGDNHVAVDLIVQH
AT1G55810     SSMEKLRKGQAYDIPNYDFKSYKNNVFPPRRVYNPSDVIILEGILIFHDPRVRDLMNMKIFVDADADYRLARRIKRDTYEKGRDIATVLDQYSKFVKPAFEDFILPTKKYADIIIPRGGDNHVAIDLIVQH
AT3G27440     SCMEKLRSGQPVNIPSYDFKIHQ-SIESSSPVNPGDVIILEGILVLNDPRVRDLMNMKIFYDTDADYRLSRRIQRDTYERGRNIQNYLEQYTKFVKPSFDEYIQPSMKYADIIIPRGGDNDVAIDLIVQH
AT4G26510     SCMEKLRQGQAVDIPKYDFKTYRSSV--FRRVYNPTDVIILEGILLFHDPRVRKLMNMKIFVCTDADYRLARRIKRDTYENGRDIGTVLDQYSKFVKPAFDDFILPTKKYADIIIPRGGDNHVAIDLIVQH
AT3G53900                                               MACSIGNAFRCSSDTLRFAPR-----QQCSSRLNPNPSSFL--SFNSSPILAQNLGASSSSLSRRTIR
Consensus     .c.e.l..gq...ip.ydfk.........r.vn..dviilegil.fhd.rvr.lmnmkifv.tdad!rlarRi.rDTve.grd...vl#QyskfvkPafdd%.lpskkyad!iiprgGdnhva.dlivqh

              261       270       280       290       300       310       320       330       340       350       360       370       380       390
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
AT5G40870     IHTKLGQHDLCKIYPN--YYVIQSTFQIRGMHTLIREKDISKHDFVFYSDRLIRLVY-EHGLGHLPFTEKQVYIPTG-AVYTGVDFCKKLCGVSIRSGESMENALRACCKGIKIGKILIHRDGDNGKQL
AT3G27190     IHTKLGQHDLCKIYPN--VFVIETTFQIRGMHTLIREKDISKHDFVFYSDRLIRLVY-EHGLGHLPFTEKQVVIPTG-SVYSGVDFCKKLCGVSVIRSGESMENALRACCKGIKIGKILIHRDGDNGMQL
AT1G55810     IHTKLGQHDLCKIYPN--LYVIQSTFQIRGMHTLIRDSKTTKHDFIFYSDRLIRLVY-EHGLGHLPFTEKQVVIPTG-SVYSGVDFCKKLCGVSVIRSGESMENALRACCKGIKIGKILIHREGDNGQQL
AT3G27440     IRTKLCQHNLCKIYSN--IFIISSTFQIKGMHTLIRDINTTKHDFVFYADRLIRLVY-EHGLGHLPFTEKQITTPTG-SVYTGVDFCKRLCGVSIRSGESMENALRACCNGIKIGKILIHRENNDGRQL
AT4G26510     ICTKLGQHDLCKIYPN--LYYIHSTFQIRGMHTLIRDSQTTKHDFVFYSDRLIRLVY-EHGLGHLPFTEKQYITPTG-CVYSGVDFCKRLCGVSVIRSGESMENALRACCKGIKIGKILIHREGDNGQQL
AT3G53900     ARTKMAASEASINGSNRHLVFVPPHPLIKHWISYLRNEQTPCPYFRNAIRELGRLLMHYEASREWLPTVYGEIMSPMGPASYEFIDPREPIAYVPILRAGLALAEHASSVLPANKIYHLGVSRDEKTLLPS
Consensus     i.TK$gqh#lckiypN..l.v!.stfqIrgmhtliR#.qt.khdFvfysdrLiRLvv.EhglghLPftek#!.tPtG..vy.g!Dfck.lcgVs!iRsGes#e#alracckgiKIgkil!hR#gdng.ql

              391       400       410       420       430       440       450       460       470       480       490       500       507
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+--------|
AT5G40870     IY-EKLPHDISER-HVLLLDPVLATGNSANQAIELLIQKGVPEAHIIFLNLISAPEGIHCVCKRFPALKIYTSEIDQCLWQEFRVIPGLGEFGDRYFGTDEEDQ
AT3G27190     IY-EKLPSDISER-HVLLLDPVLGTGNSANQAIELLIQKGVPEAHIIFLNLISAPEGIHCVCKRFPKLKIYTSEIDQCLNEEFRVIPGLGEFGDRYFGTDE
AT1G55810     IY-EKLPSDISER-HVLLLDPILGTGNSAVQAIRLLISKGVPESNIIFLNLISAPEGYNVVCKKFPRIKIYTSEIELGLNDEFRVVPGMGEFGDRYFGTDDE
AT3G27440     IY-EKLPKDISSR-HVFLLDPVLASGYSAVKAITLLLSKGVPESHIIFLNLIAAPQGIHALCKKFPMLKIYTSEIDSSLNEDSRVIPGLGEFADRYFGTNNINSKVSSLSTNLKLRS
AT4G26510     VY-EKLPHDISER-HVLLLDPILGTGNSAVEAINLLISKGVPEGNIIFLNLISAPQGVHVVCKKFPRIKIYTSEIDNGLNEEFRVIPGMGEFGDRYFGTDDD
AT3G53900     VYLNKLPDEFPKNSRVFLVDPVLATGGTIMAAMDLLKERGLSVQQIKVICAIAAPPALSKLNEKFPGLHVYAGIIDPEVNEKGYIIPGLGDAGDRSFGTETHWVK
Consensus     !Y.#KLP.#iser.hVlLlDP!LatGnsa..Ai.LLi.kGvpe..IiflnlIsAP.g.h.vckkFP.lk!vtseI#..lN#efr!!PG$G#fgDRyFGT#................
```

Figure 4.8 Amino acid sequence alignment of *Arabidopsis* UPRTases.

The functional characterization of a gene encoding UPRT in *Arabidopsis* was reported by Islam et al. (2007). This report indicated that *UPRT5* has a dual role acting as a uridine kinase and also a uracyl phosphoribosyltransferase to produce UMP through the pyrimidine salvage pathway in *Arabidopsis*. Plants produce toxic secondary metabolites derived from pyrimidines for use as defence compounds (Kafer et al., 2004), this may indicate a role for pyrimidine metabolism in disease signalling.

80% of the secondary metabolites identified by organic chemists are produced by plants. Most of these compounds have a role in defence to combat bacteria, fungi, insects, or even other parasitic plants. Chief among these secondary metabolites are the terpenes and phenolic compounds, however secondary metabolites from pyrimidines have also been described (Kafer et al., 2004). Plants of the Mimosa family synthesize 5-aminouracil as a unique defence compound (Brown and Turan, 1995). This compound blocks the mitotic cycle and inhibits incorporation of guanosine into nucleic acids. This function makes 5-aminouracil as a uniquely useful cell cycle inhibitor (Oliev, 1994). Other secondary metabolites, like lathyrine, have antimicrobial activity and act as cytokinin mimics in some plants (Kafer et al., 2004).

In order functional characterise the role of DIN1 in plants defence, we performed a loss of function assay. T-DNA of *Agrobacterium tumefacians* were used to generate loss of function mutations in *Arabidopsis* (Radhamony et al., 2005). In this study we have successfully isolated heterozygote mutant lines. In the next stage, the seeds from these lines can be used to screen for *DIN1* KO homozygous lines and investigate the role of *DIN1* in plant defence in the following generations.

It has been reported by Islam et al., that GFP-UPRT5 fusion protein is present in the cytoplasm (Islam et al., 2007). It would be worthwhile to investigate the DIN1 protein expression at the cellular level. In order to study the subcellular localization of DIN1, I have generated DIN1-GFP fusion constructs. Using GFP-DIN1 fusion constructs, it would be insightful to see if DIN1 localization changes during disease signalling.

Considering the time frame of my study, I had to stop this study at this stage. There are numerous avenues for further investigations into DIN1 function. The findings in this chapter serve as an excellent starting point for further study into this undoubtedly strongest interactor of UBP12.

CHAPTER 5

In vitro ubiquitin activity assays of *Arabidopsis thaliana* ubiquitin and its various mutant isoforms

5.1 Introduction

E1, E2 and E3 ezymes are involved in the ubiquitin (Ub) conjugation cascade. In the initial reaction, E1 activates the Ub by utilising energy from ATP hydrolysis to mediate the formation of an E1-Ub intermediate in which the C-terminal glycine of Ub is linked via a thiolester bond to the E1. Activated Ub is then transferred to an E2 by a transesterification reaction. This E2-ubiquitin conjugate transfers the Ub moiety to the substrate, usually using an E3 as the catalyst (Vierstra, 2003). E3 enzymes impart substrate recognition to the process and either promotes direct transfer of Ub to substrates from E2 or from a final E3-Ub intermediate prior to transfer (Vierstra, 2004).

After attachment of an initial ubiquitin moiety to a substrate, additional Ub are ligated to specific internal lysine residues on the first Ub to form poly-Ub chains. Poly-Ub chains of at least four ubiquitin moieties (tetra-ubiquitin) are required to provide an efficient proteasome delivery signal (Thrower et al., 2000). While K48 linked chains usually (but not always) signal proteasome proteolysis, non-proteolytic signalling by K63 linked poly-Ub chains has been shown to mediate DNA repair, trafficking and kinase activation (Fushman and Pickart, 2004). Although chains linked through K29 and K6 have been observed, their precise function is not currently clear (Fushman and Pickart, 2004). Structure or function analysis of various amino acid substitutions in the *Arabidopsis thaliana* ubiquitin and their role in the ubiquitin cascade have not been studied before.

This chapter reports the *in vitro* demonstration activity of a *Arabidopsis thaliana* ubiquitin and its various mutant isoforms (corresponding lysine substitutes to arginine). I also report the successful production of rabbit polyclonal antibody to plant ubiquitin, in collaboration with Agrisera company.

5.2 *In vitro* activity assay of *E. coli* expressed *Arabidopsis thaliana* ubiquitin protein and its various mutant isoforms

5.2.1 Expression and purification of *Arabidopsis thaliana* ubiquitin and various mutant isoform from *E. coli*

To demonstrate the activity of *Arabidopsis thaliana* ubiquitin (*AtUbq11*) and 3KR (K29, K48, K63 triple mutant), K29, K48, K63 (various mutant isoforms), Histidine tagged fusion constructs corresponding to AtUbq11 and various mutant isoforms were generated. His tagged AtUbq11 and the various mutant isoforms were made using pET30a expression vector (Hou et al., 2006). The pET-30a HA-*AtUbq11* and various mutant isoforms were provided by R. Taylor (Ph.D student in Sadanandom group). Amino acid sequence alignment of HA-*AtUbq11* and various mutant isoforms are shown in figure 5.1.
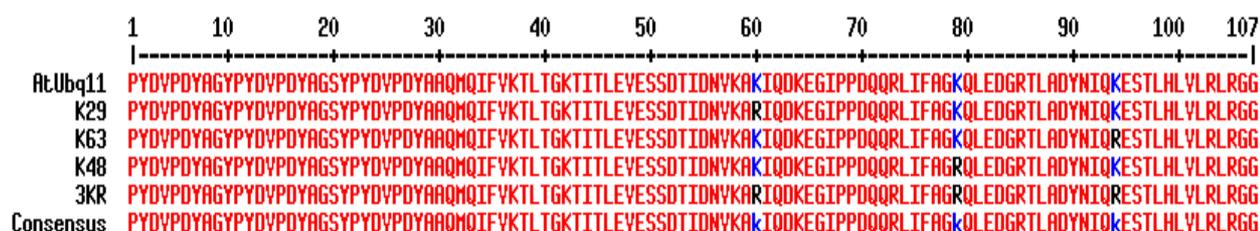
Figure 5.1 Amino acid sequence alignment of HA tagged *AtUbq11* and various mutant isoforms.

Alignment colour indicates high conservation (red), low conservation (blue), no conservation (black).

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AtUbq11 and its various mutant isoforms were transformed into *E. coli* strain BL21 (DE3) for protein expression. The BL21 (DE3) *E. coli* cells were grown in 500 ml cultures and induced with 1M IPTG at an OD_{600} of 0.65. After a two hour expression period at 37°C, cells were recovered by centrifugation and soluble extracts were prepared using BugBuster cell lysis solution (Mey et al., 2008). Protein extracts prepared from both induced and uninduced cells were analysed by SDS-PAGE to confirm the expression of AtUbq11 and its various mutant isoforms.

As AtUbq11 and the various mutant isoforms were fused with a HA tag (2kDa), the calculated molecular weight of HA-AtUbq11 and various mutant isoforms is fused to the 2.5 kDa His tag is ~13 kDa. The distinct band present at ~13 kDa in Induced total (IT) and Induced Soluble (IS) fraction in the SDS-PAGE (figure5.2A) indicates the expression of the protein. The presence of 6× His tagged AtUbq11 and its various mutant isoforms was confirmed by performing an immunoblotting experiment using anti-His antibody (figure 5.2B).

(A).

1.

UT   U   U   IT   IS   II

_____   _____
Uninduced   Induced

2.

UT   US   UI   IT   IS   II

_____   _____
Uninduced   Induced

3.

UT   US   U   IT   IS   II

_____   _____
Uninduced   Induced

4.

UT   US   UI   IT   IS   II

_____   _____
Uninduced   Induced

5.

UT   US   UI   IT   IS   II

_____   _____
Uninduced   Induced
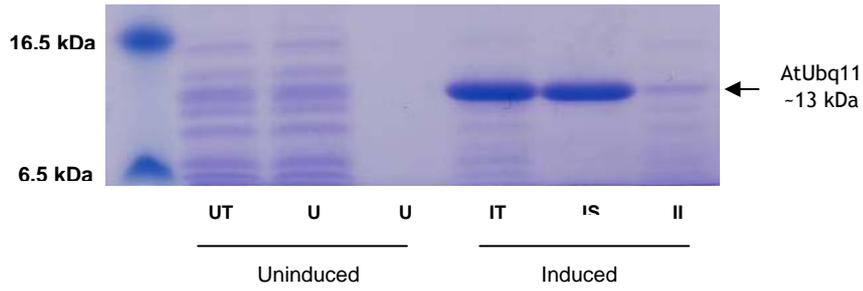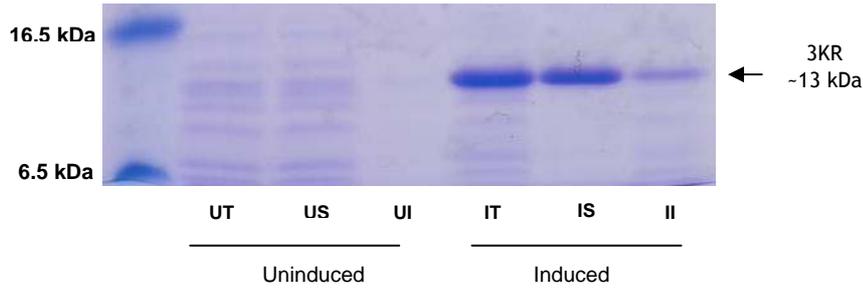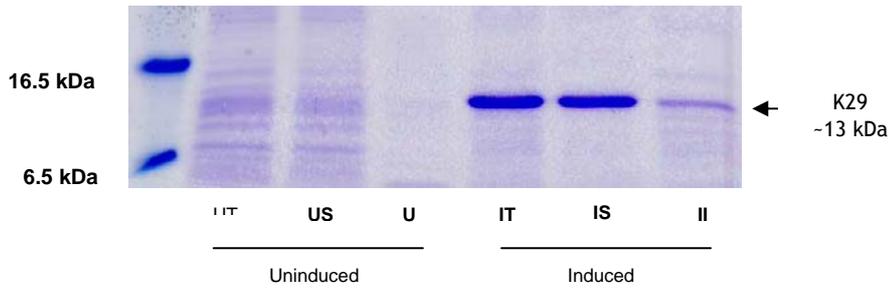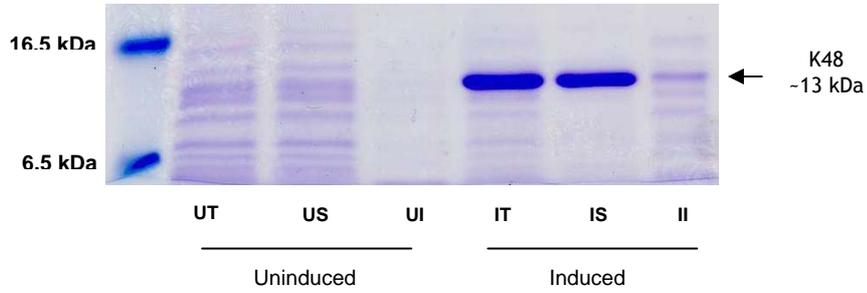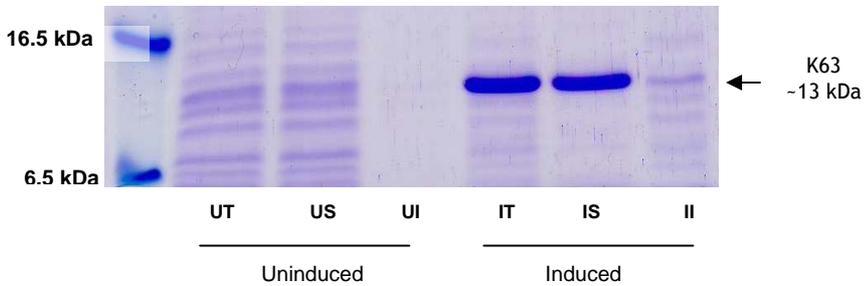
(B).
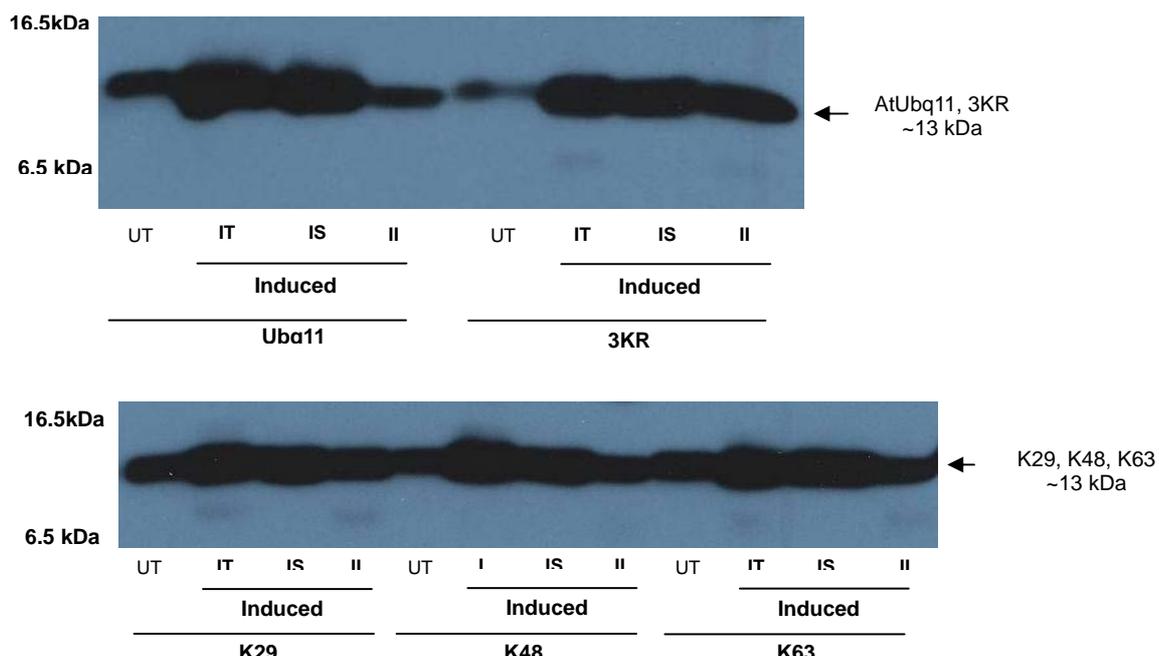
Figure 5.2 Generation of 6× His tagged HA-AtUbq11 and its various mutant isoforms.

(A). Comassie stained 13% SDS-PAGE used for the analysis of following fusion proteins (1) AtUbq11, (2) 3KR, (3) K29, (4) K48, (5) K63.
The lanes following the molecular weight marker are as follows Uninduced total (UT), Uninduced soluble (US), Uninduced insoluble (UI), Induced total (IT), Induced soluble (IS), Induced insoluble (II).
(B). Immunoblotting analysis of 6× His tagged AtUbq11 and various mutant isoforms expression.
The proteins were separated on 13% SDS-PAGE and transferred onto a PVDF membrane. The free space on PVDF membrane was blocked with 5% milk for 1 hour followed by incubating in anti-His primary antibody (1:2000) for 2 hours followed by further incubation with anti-Rabbit secondary antibody coupled with HRP (1:12.000) for 1 hour. The presences of proteins were visualized using standard ECL detection kit. The presence of distinct bands at ~13kDa confirms the expression of various forms of ubiquitin. The UT, IT, IS, II terms in the exposed film refer to uninduced total, induced total, induced soluble and induced insoluble respectively.

The soluble 6× His tagged AtUbq11 and its various mutant isoforms were purified on a nickel affinity chromatography column. The proteins were eluted from the column in 1ml fractions using high imidazole buffer. Eluted proteins were dialyzed overnight in Tris-HCl (pH 7.5) buffer. The following SDS-PAGE showed the eluted fractions (figure 5.3).
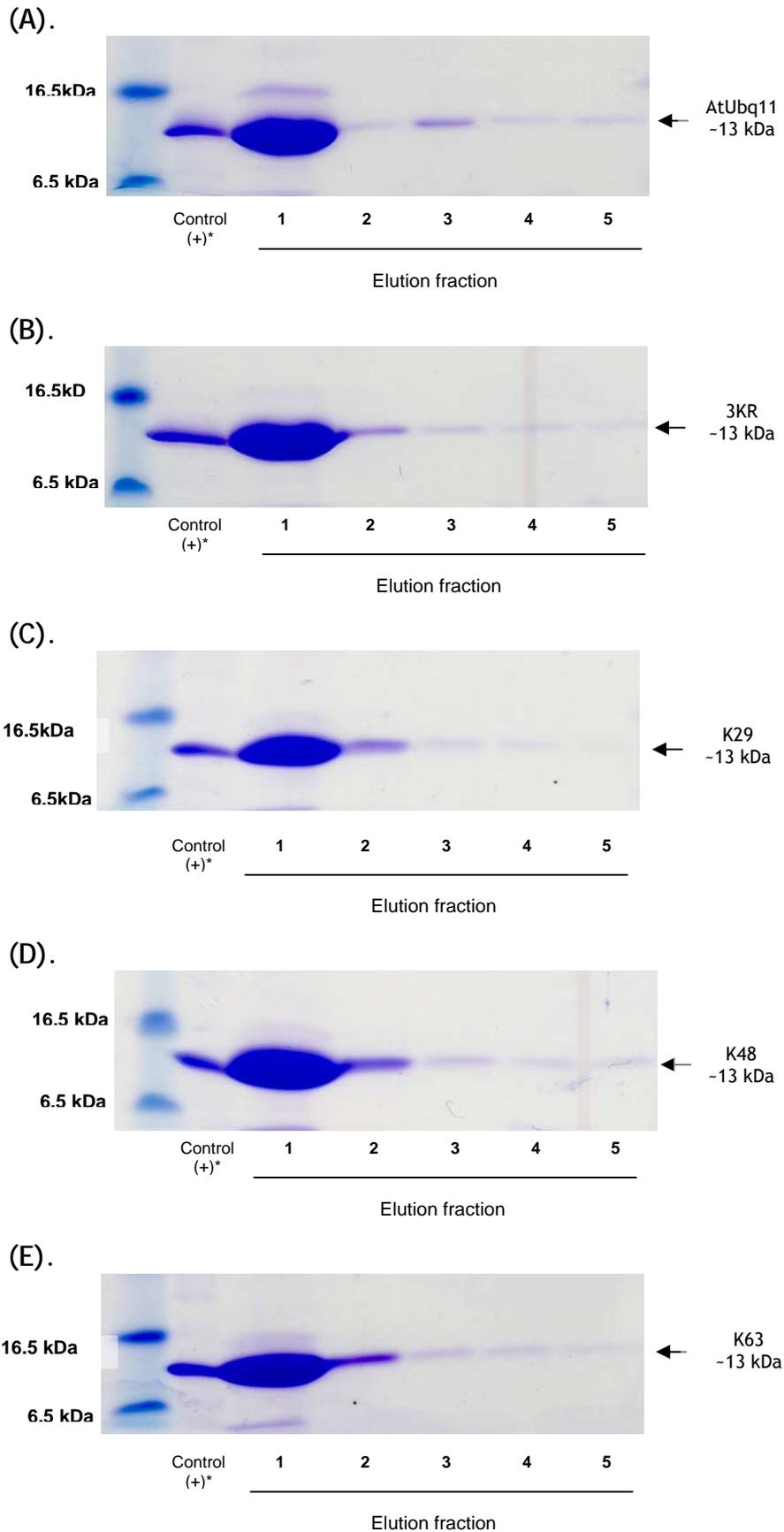
Figure 5.3 Purification of soluble 6×His tagged AtUbq11 and various mutant isoforms.

Comassie stained 13% SDS-PAGE showing purified soluble 6× His tagged of HA AtUb and various mutant isoforms. (A) AtUbq11, (B) 3KR, (C) K29, (D) K48, (E) K63. *As a positive control the crude lysate were used.

5.2.2 Anti-Ubiquitin antibody is reactive against plant and human ubiquitin

In order to test the affinity of plant ubiquitin antibodies against ubiquitin from other species we ordered Agrisera, Sweden to produce ubiquitin antibodies for HA AtUbq11. HA AtUbq11 protein purified by affinity chromatography was given to the antibody manufacturer. 200 µg of the purified protein was injected into a Rabbit on the first day followed by an injection 100 µg on the seventh day. In order to verify rabbit is a suitable animal to develop antibody serum prior to immunization was collected (figure 5.4 A). Serum was collected every week, the serum delivered after fourth immunisation (fourth bleed) were used in this study. The antibodies from the serum were purified and tested by performing immunoblotting (figure 5.4B). Afterwards, this anti-Ub antibody was used for all part of this thesis.
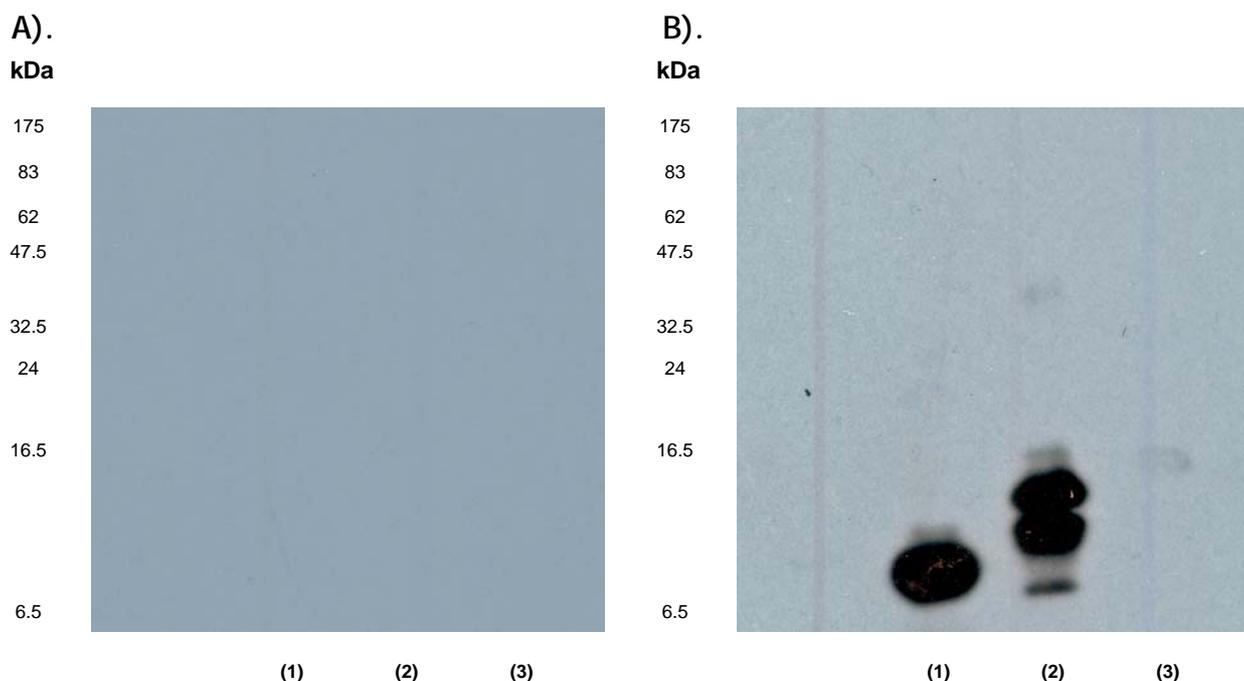
Figure 5.4 Western blot analysis of ubiquitin protein from various species.

(A) 5 µl of recombinant protein from Human (1), *Arabidopsis thaliana* His-tagged ubiquitin (2), *Arabidopsis thaliana* His-tagged SUMO (3) was separated on 13 % gel and blotted on PVDF membrane. Filters were blocked in 5% milk for (1h), incubated with 1:10.000 anti-Ubiquitin preimmune antibody (2h) followed by incubation with 1: 12.000 secondary anti-rabbit (1h) coupled with HRP and visualization (10 seconds exposure) with standard ECL.
(B) 5 µl of recombinant protein from Human (1), *Arabidopsis thaliana* His-tagged ubiquitin (2), *Arabidopsis thaliana* His-tagged SUMO (3) was separated on 13 % gel and blotted on PVDF membrane. Filters were blocked in 5% milk for (1h), incubated with 1: 10.000 anti-Ubiquitin 4th bleed antibody (2h) followed by incubation with 1: 12.000 secondary anti-rabbit (1h) coupled with HRP and visualization (10 seconds exposure) with standard ECL.

The western blot (figure 5.4) indicates that rabbit is a suitable animal to develop anti-Ub antibody, as there were no bands produced in preimmune sample. The anti-Ub antibody produced was able to detect human ubiquitin and *Arabidopsis* ubiquitin but not *Arabidopsis* SUMO (S̲mall U̲biquitin like M̲o̲difier).

5.2.3 *In vitro* ubiquitin assay of *E. coli* expressed AtUb and various mutant isoforms

The ubiquitination assays were performed to test whether mutant forms of plant Ub can affect ubiquitination *in vitro*. Purified fractions of AtUbq11 and various mutant isoforms was incubated along with yeast E1, human E2 (UbcH5a), plant E3 (PUB-17) at 37°C for 1 hour. The reactions were analysed by SDS-PAGE and immunoblotting.

In vitro ubiquitination were performed to study the effects of mutations. The ubiquitination pattern of ubiquitin mutant forms did not show any considerable difference in the pattern of *in vitro* ubiquitination compared to the wild type ubiquitin.

	1	2	3	4	5	6	7	8	9	10
E1	+	+	+	+	+	-	+	+	+	+
E2	+	+	+	+	+	+	-	+	+	+
E3	+	+	+	+	+	+	+	-	+	+
His-Ubq11	-	-	-	-	+	-	-	-	-	-
His-3KR	-	-	-	+	-	-	-	-	-	-
His-K29	+	-	-	-	-	-	-	-	-	-
His-K48	-	+	-	-	-	-	-	-	-	-
His-K63	-	-	+	-	-	-	-	-	-	-
His-Human	-	-	-	-	-	+	+	+	-	+
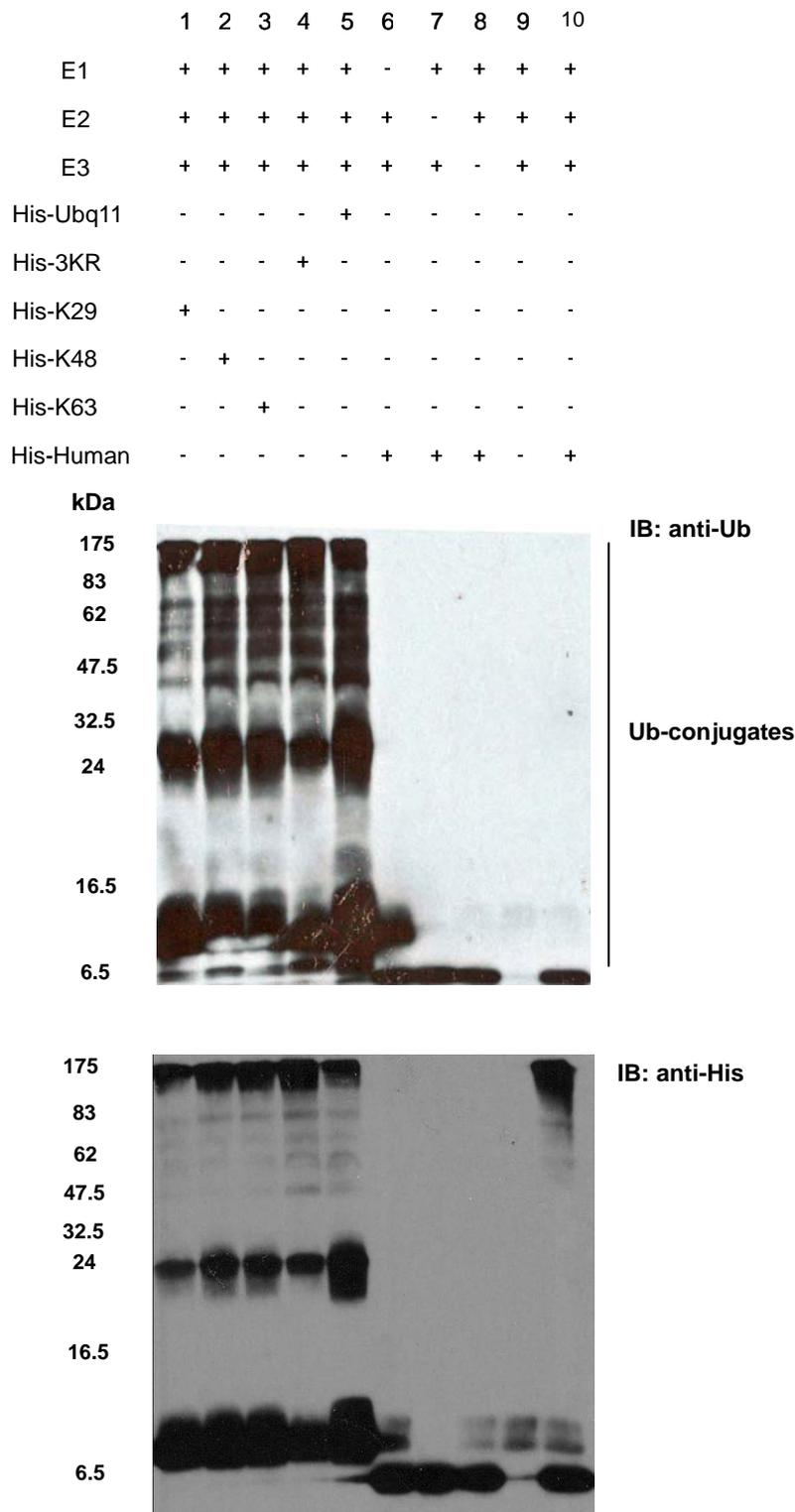
Figure 5.5 *In vitro* ubiquitination assays of Ubq11 and various mutant isoforms

Ubiquitination assays with Ubq11 and various mutant isoforms. Ubq11 and various mutant isoforms were expressed and purified from *E. coli* and tested for ubiquitination activity in the presence of yeast E1, human E2 (UbcH5a), plant E3 (PUB17). The immunoblots were probed with anti-Ub antibody (1:10.000 for 2h) (top panel) to detect ubiquitinated *E.coli* proteins. Anti-His antibody (1:2.000 for 2h) was used to detect His-Ubq11 and various mutant isoforms. As control for ubiquitin activity we used human ubiquitin. The various components of the reactions were indicated by, + which indicates the presence and - which indicates the absence.

5.3 Discussion

The Ubiquitin protein is one of the most conserved proteins in nature. Most frequently, the ubiquitin tag is used to mark proteins for proteolytic degradation and recent studies have shown that it can also be used for non proteolytic functions (Hocstrasser, 2000). In the cell proteins that are tagged with K48 linked ubiquitin chains are targeted for degradation by the 26S proteasome (Chau et al., 1989). Recent data indicate that variation in ubiquitin chain length and structure can result in differint signalling outcomes (Pickart and Fushman, 2004). Proteomic approaches to study ubiquitination patterns in yeast (Peng et al., 2003) have reported the presence of distinct ubiquitin chains linked through all seven available lysine residues (K6, K11, K27, K29, K33, K48 and K63). Biochemical and structural data suggest that distinct ubiquitin chain topologies adopt different three-dimensional conformations and differences between these chain structures are presumed to confer distinct signalling outcomes (Pickart and Fushman, 2004). In addition to the K48 linkage, the best characterised alternative ubiquitin chain topologies are linked through lysines K63 and K29. In mammalian cells, K63 linked ubiquitin has been implicated in numerous signalling processes that include DNA damage tolerance and protein trafficking (Zapata et al., 2001). Recent data indicates that K29 linked ubiquitin chains function in lysosomal protein degradation where K29 tagged substrates are targetted to lysosomes via the Ubiquitin Fusion Degradation (UFD) pathway (Chastagner et al., 2006).

In vitro ubiquitination assays of Ubq11 and various mutant isoforms

The effects of various amino acid substitutions in the *Arabidopsis thaliana* ubiquitin and their role in the ubiquitin cascade have not been studied before. In this study, I studied the effects of three substitutions at the following positions K29, K48, K63 and 3KR (a combined triple mutant of K29, K48 and K63) in the *Arabidopsis thaliana* ubiquitin gene.

The genes encoding the mutant forms of ubiquitin were cloned into a pET expression sysem. *In vitro* ubiquitination were performed to study the effects of mutations. The mutant forms of ubiquitin did not show any considerable difference in the pattern of in vitro ubiquitination compared to the wild type

ubiquitin. Ubiquitin protein has 7 lysine molecules together, where as in my studies, I used only 3 mutant forms of ubiquitin. The similarity in the ubiquitin might be due to the presence of other lysines which still can act as active sites for binding to ubiquitin conjugating enzymes. In order to study analyse the properties of individual lysine residue, further study can be extended by producing multiple lysine mutants (i.e. deleting substituting all the lysines with an exception of one lysine, 6KR) to see any difference in ubiquitination properties.

Development of anti-Ub antibody

The purified protein of wild type ubiqutin was used to develop antibodies. The antibody when used to detect various ubiquitins i.e AtUbq11, human ubiquitin, and SUMO protein (as a negative control), the antibody showed affinity only for AtUbq11 ubiquitin and human ubiquitin. Since anti-Ub antibody was not reactive to SUMO this antibody is specific to ubiquitin only and it might be reactive against ubiquitin from various species. Further studies can be performed to verify the antibody specifity to ubiqutin from other species (i.e yeast, rat, etc) and develop specific antibody for different organisms.

CHAPTER 6

Final discussion

6.1 Introduction

Ubiquitin mediated proteolysis is a central regulatory mechanism in the control of several cellular processes in yeast and animals. The ubiquitin-26S proteasome system has also been implicated in growing number of plant signalling pathways, including those mediating responses to hormone, light, sucrose, developmental cues and plant defence (Devoto et al., 2003). The identification of SGT1 and COP9 as essential components for R gene-mediated disease resistance suggests that ubiquitin plays an important role in plant defence (Gray, 2002) (Devoto, et al., 2003). Recently, based on microarray data Kim et al., suggest that UBP6 and UBP12 may play a role in the defence response (Kim et al., 2006).

The primary aim of this study was to establish the potential involvement of AtUBP12 and AtUBP13 in plant defence. The role of *UBP12* and *UBP13* in *Arabidopsis thaliana* defence signalling was studied by performing loss of function assays. RNAi approaches were taken to study *AtUBP12* and *AtUBP13* which established that *AtUBP12* and *AtUBP13* are a negative regulator of plant defence. The solanaceous AtUBP12 ortholog, NtUBP12 was identified and transient gain of function approaches were employed to examine its role in disease resistance signalling. Using transient overexpression it was demonstrated that solanaceous UBP12 proteins function as negative regulators of the Cf-9 triggered HR. In this study added to the above mentioned objective I tried to find the interactor of UBP12 protein, analyse ubiquitination pattern using mutant ubiquitin forms and finally ubiquitn antibodies were produced and analysed.

6.2 AtUBP12 and AtUBP13 play a critical role in disease resistance

Ewan (2008) showed that *ubp12* and *ubp13* double knock out (KO) mutants are embryo lethal. Therefore using gene silencing approach is a viable option to evaluate the role of *AtUBP12* and *AtUBP13* in plant disease resistance. Bacterial resistance assays in *AtUBP12 and AtUBP13* silenced lines indicated significant difference in resistance between Col-0 control and UBP_RNAi during virulent *Pst* DC3000 infection. This result indicates that *AtUBP12* and *AtUBP13* are a negative regulator of defence response. This outcome is supported by the finding that transient overexpression of AtUBP12 in the hetrologous tobacco system supresses the HR induced by the *C. fulvum* avirulence factor Avr9. The ability of AtUBP12 to function in a transgenomic functional assay suggests that it is a conserved signalling component of plant disease resistance and that overexpression of either AtUBP12 or AtUBP13 in *Arabidopsis* my also yield novel HR associated phenotypes.

The developmetal phenotypes observed in *Arabidopsis AtUBP12* and *AtUBP13* silencing lines clearly indicate that both proteins stabilise common targets and sets a precedent for their potential redundancy in other signalling pathways. Chromosome duplication events have shaped the *Arabidopsis* genome extensively (Blanc et al., 2003) and frequently result in masking of potential phenotypes due to genetic redundancy between closely related genes such as *AtUBP12* and *AtUBP13* (Pickett and Meeks-Wagner, 1995).

Since RNAi silencing efficiency of UBP12 and UBP13 was reduced on the following generation, future functional studies could be conducted on transgenic plants which are dexamethsone inducible *UBP12* or *UBP13* RNAi (Wielopolska et al., 2005) to induce silencing of *AtUBP12* or *AtUBP13*. The generation of such stable lines may be essential to clarify the association of *AtUBP12/AtUBP13* with disease resistance signalling, providing a robust alternative system to the Solanaceae based transient gain and loss of function approaches described in this study. Ultimately, efficient *AtUBP12/AtUBP13* loss of function lines would be of value to characterise their role in plant pathology as *Arabidopsis* provides a model system to examine plant

interactions with a variety of fungal, viral and bacterial pathogens (Kunkel, 1996). This study can be further extended to investigate the role of UBP enzymes and ubiquitination cascade in defence signalling pathways in Arabidopsis thalina.

6.3 DIN1 as an interactor of UBP12

Results reported in this study have shown DIN1 as an interactor of UBP12. In Arabidopsis, *DIN1* is showed to be involved in the synthesis UMP from phosphoribosyl phyrophosphate (PRPP) (Andersen et al., 1992) (Islam et al., 2007).

Plants produce toxic secondary metabolites derived from pyrimidines which are used in combating pathogens (Kafer et al., 2004), this evidence may indicate a role for pyrimidine metabolism in disease signalling. 80% of the secondary metabolites identified by organic chemists are produced by plants. Most of these compounds serve a defensive function to combat bacteria, fungi, insects, or even other parasitic plants. Chief among these secondary metabolites are the terpenes and phenolic compounds, mainly derived from pyrimidines (I.e. 5-aminouracil, lathyrine, etc) (Kafer et al., 2004). This evidence strongly indicates the role of UBPs and protein interacting with ubps such as Din1 to actively participate in various pathways linked to defence signalling.

In this study, we have successfully isolated a heterozygote line and generated DIN1-GFP tagged construct. Future studies could be conducted to establish the potential involvement of *DIN1* in plant defence using gain function assay using this DIN1-GFP tag construct. Using the heterozygotes lines we have isolated, the role of DIN1 in disease resistance against virulent and avirulent strains of *Pseudomonas syringae* can also investigated. The sub cellular localisation of DIN1 can be visualised by detecting the fluorescence of GFP fused to DIN1 and this might reveal intricacies of plant pathogen interactions and their specific locations.

6.4 *Arabidopsis thaliana* ubiquitin and its various mutant isoforms.

The mutant forms of ubiquitin did not show any considerable difference in the pattern of *in vitro* ubiquitination compared to the wild type ubiquitin. Ubiquitin protein has 7 lysine residues which can actively participate in formation of thiol ester bond and tagging of proteins for degradation, whereas in my studies, I used only 3 lysine mutations. Due to the presence of other lysines which can still serve as active sites ubiquitin the ubiquitin assays I performed did not show any variation in the ubiquitination pattern. The in vitro conditions employed might also affect the ubiqutination pattern. This study can be further extended by generating mutations for all the lysine residues and study ubiquitination pattern in vivo and in vitro, the study might reveal the importance of various lysine residues and the fate of targets when attached to different lysine residues.

In this study we have successfully generated a plant anti-ub antibody which is able to recognize against plant and human ubiquitin. This antibody is specific to ubiquitin only, because it can not detect SUMO. Future studies could be conducted to verify antibody specifity to ubiquitin from other species.

6.5 Conclusions

This study details several novel findings that contribute to existing knowledge of plant deubiquitinating enzymes.

AtUBP12 and *AtUBP13* are closely related paralogs that function redundantly to regulate plant development since double knockouts are lethal. Gene induction data suggests that *AtUBP12* and *AtUBP13* may also function to regulate disease resistance and this hypothesis is supported by the finding that RNAi lines of AtUBP12 and AtUBP13 was more resistant to virulent *PstDC3000.*

Sequence analysis suggests a conserved domain for substrate interaction between plant UBP12 proteins and its human ortholog HAUSP7. This finding may be of use in future attempts to identify the biological substrates of plant UBP12 proteins and provide an insight into the mechanism by which UBP12 proteins are transported to the nucleus.

DIN1 is an interactor of UBP12, which may play a role in synthesis of secondary metabolites for defence compounds and this may have a broader implications in the regulation of plant defence against pathogens.

In order to study the ubiquitination properties of ubiquitin lysine it is worthwhile to generate multiple lysine mutants (i.e. deleting substituting all the lysines with an exception of one lysine, 6KR).

The reported results present a novel association of ubiquitin pathway with plant disease resistance signalling which may have broader implications for our current understanding of fungal and viral resistance in plants.

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APPENDICES

Appendix 1

pGEM®-T Easy vector map
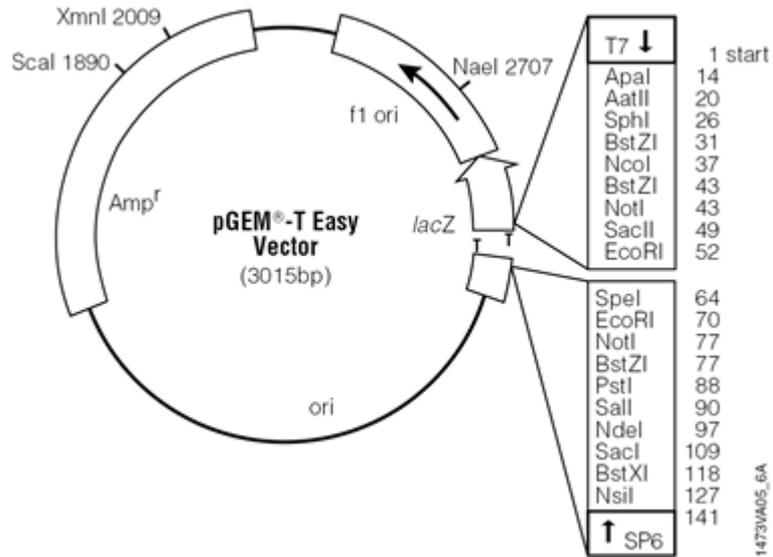
Below is the multiple cloning site pGEM®-T Easy vector. Restriction sites are labelled to indicate the cleavage site.
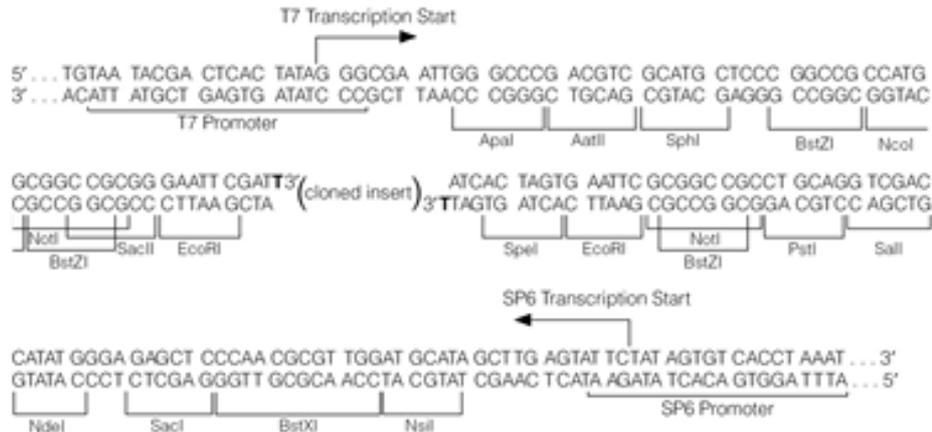
pENTR^{TM}/D-TOPO map
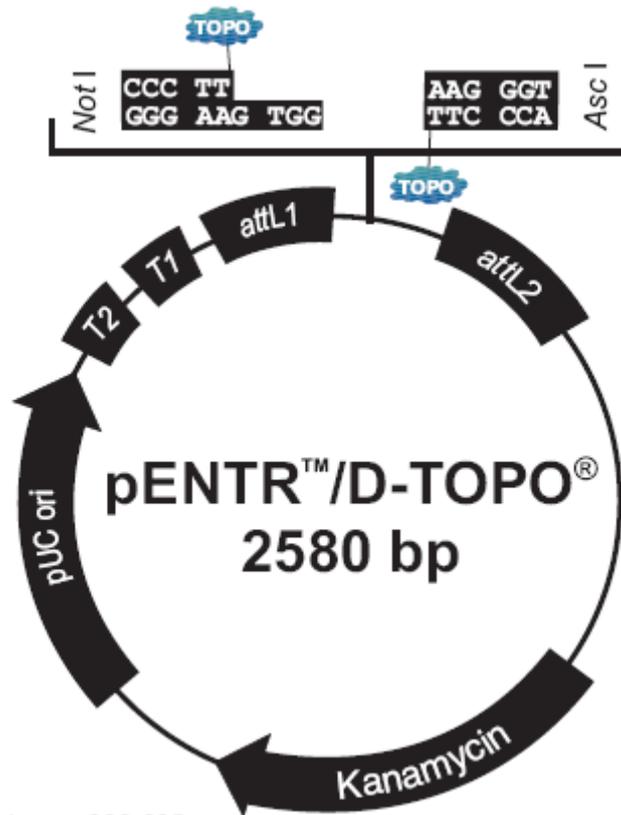
Below is the multiple cloning site of pENTR^{TM}/D-TOPO. Restriction sites are labelled to indicate the actual cleavage site. Shaded regions correspond to those DNA sequences transferred from the entry clone into the destination vector following LR recombination.

pENTR™4 vector map

Dra I* | Xmn I** | Sal I | BamH I | Kpn I | EcoR I | ccdB | EcoR I | Not I | Xho I | EcoR V

T1 T2 attL1 attL2

pENTR™

Kanamycin

pUC ori

Below is the multiple cloning site of pENTR™4. Restriction sites are labelled to indicate the cleavage site. Shaded regions correspond to those DNA sequences transferred from the entry clone into the destination vector following recombination.

```
                                                 attL1
352  GGGCCCCAAA TAATGATTTT ATTTTGACTG ATAGTGACCT GTTCGTTGCA ACAAATTGAT
     CCCGGGGTTT ATTACTAAAA TAAAACTGAC TATCACTGGA CAAGCAACGT TGTTTAACTA

412  AAG CAA TGC TTT TTT ATA ATG CCA ACT TTG TAC AAA AAA GCA GGC TCC
     TTC GTT ACG AAA AAA TAT TAC GGT TGA AAC ATG TTT TTT CGT CCG AGG
     Nco I                Xmn I        Sal I      BamH I         Kpn I  EcoR I
460  ACC ATG GGA ACC AAT TCA GTC GAC TGG ATC CGG TAC CGA ATT CGC --- ccdB gene ---
     TGG TAC CCT TGG TTA AGT CAG CTG ACC TAG GCC ATG GCT TAA GCG
     EcoR I       Not I        Xho I        EcoR V
918  TAG AAT TCG CGG CCG CAC TCG AGA TAT CTA GAC CCA GCT TTC TTG TAC AAA
     ATC TTA AGC GCC GGC GTG AGC TCT ATA GAT CTG GGT CGA AAG AAC ATG TTT
                                         attL2
969  GTTGGCATTA TAAGAAAGCA TTGCTTATCA ATTTGTTGCA ACGAACAGGT CACTATCAGT
     CAACCGTAAT ATTCTTTCGT AACGAATAGT TAAACAACGT TGCTTGTCCA GTGATAGTCA
                                                          reverse primer binding site
1029 CAAAATAAAA TCATTATTTG CCATCCAGCT GCAGCTCTGG CCCGTGTCTC AAAATCTCTG
     GTTTTATTTT AGTAATAAAC GGTAGGTCGA CGTCGAGACC GGGCACAGAG TTTTAGAGAC

1089 ATGTTACATT
     TACAATGTAA
```

pET-30a map

The pET-30a vectors carry an N-terminal His· Tag/thrombin/S· tag TM/enterokinase configuration plus an optional C-terminal His· Tag sequences. Unique sites are shown in the circle map below :
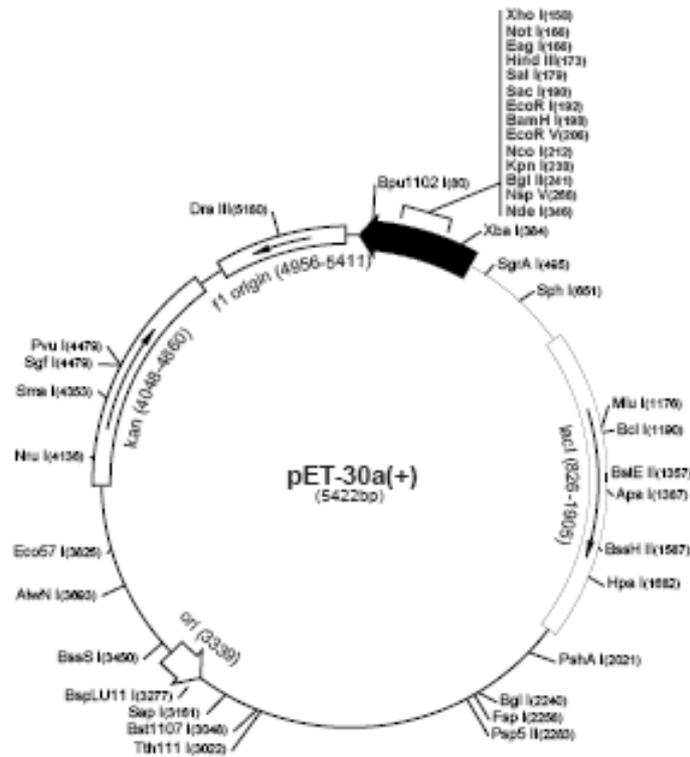
Below is the multiple cloning site pET-30a vector. Restriction sites are labelled to indicate the cleavage site.

pET-30a-c(+) cloning/expression region
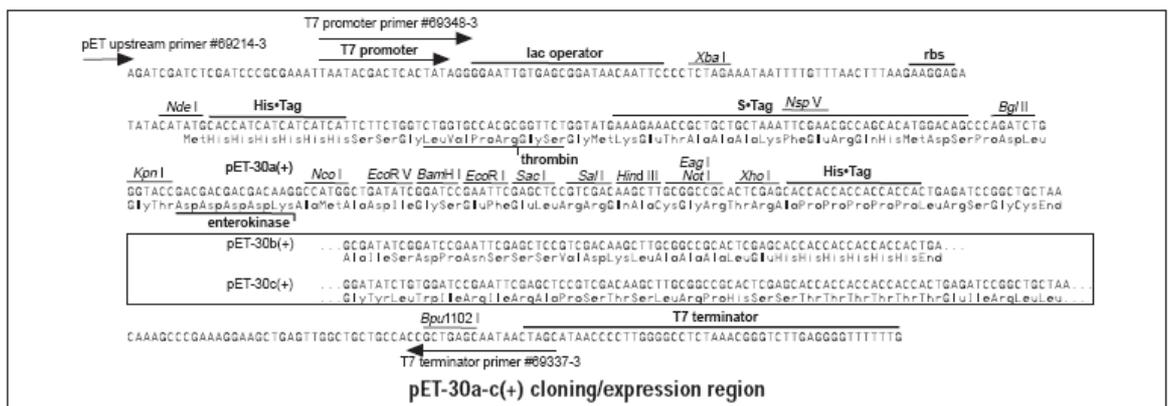
Appendix 2

Growth Medium Composition

Component	MS (Sigma M5519)
Macro Nutrient Salts (mM)	
Ammonium Nitrate	20.61
Calcium Chloride anhydrous	2.99
Magnesium Sulfate anhydrous	1.50
Potassium Nitrate	18.79
Potassium Phosphate monobasic	1.25
Sodium phosphate	
Micro Nutrient Salts (μM)	
Boric Acid	100.00
Cobalt Choride-6H2O	0.110
Cupric Sulfate-5H2O	0.10
Na_2- EDTA	100.00
Manganese Sulfate-H2O	100.00
Molybdic Acid sodium salt	1.03
Potassium Iodide	5.00
Ferrous Sulfate-7H$_2$O	100.00
Zinc Sulfate-7H$_2$O	29.91
Organics (μM)	
Glycine	26.64
myo-Inositol	560.00
Nicotinic Acid (Niacin)	4.06
Pyridoxine HCL (vitamin B$_6$)	2.43
Thiamine HCL (vitamin B$_1$)	0.30