The Role of the EAR Domain in Transcriptional Repressors involved in Plant Defence

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The Role of The EAR Domain in Transcriptional Repressors involved in Plant Defence

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Abstract

Plants respond to abiotic and biotic stressors through hormone signalling which forms an integral part of the plant immune response, and is often the target of phytopathogens. Changes in hormone levels are often underpinned by transcriptional reprogramming driven by specific transcription factors (TF) which collectively regulate the spatial, temporal expression levels of hormone biosynthetic and signalling genes in a co-ordinated manner.

This study examined two contrasting transcription repressors (TR) implicated in Arabidopsis - Pseudomonas syringae disease development; a MYB like transcription factor (HUB37) and a JAsonate Zim domain (JAZ) containing transcriptional repressors involved in jasmonate signalling. The MYB transcription factor was identified by modelling as a core hub in immune signalling, whereas JAZ5 was recently shown to co-operate with JAZ10 during transcriptional reprogramming, to restrict P. syringae growth and attenuated chlorosis.

The transcriptional repressor ERF-associated Amphiphilic Repression (EAR) domains confer dominant transcriptional repressive functions. HUB37 contains one EAR domain and JAZ5 contains 2 EAR domains.

Previous transcriptional inference modelling predicted HUB37 was a highly transcription factor that negatively regulated A. thaliana defence responses to P. syringae. This was validated by testing a HUB37 loss of function mutant (Siddharth Jayaraman, Marta de Torres per com). JAZ5 and JAZ10 are required for full immunity to P. syringae. Thus, this study sought to develop molecular and genetic tools to explore the role of the EAR domain in disease.

Golden Gate cloning and targeted mutagenesis were used to generate epitope tagged lines of JAZ5 and HUB37 with and without EAR domains. These were assembled into T-DNA transformation vectors and various transgenic lines characterised. At the end of the project we had generated a range of lines and shown that HUB37 was the target for post-transcriptional degradation by bacterial effects. Our data predicts that bacterial effectors function to remove a negative regulator of plant immunity to promote disease.
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Genna Davies and Clara Rodriguez Herrero for their complementary tenacious work ethic and continued friendship.

My family for supporting the difficult decision not to relocate with the team. To conclude my research and pass on the tools with scope to the next Scientist was daunting. I am happy in the knowledge that this work is ongoing and I have added to scientific research.
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L2 pICH86966 - JAZpro:HA:JAZ5gene:Act.2


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List of Abbreviations
ABA abscisic acid
bHLH basic-Helix-Loop-Helix
bp base pairs
BR brassinosteriods
dpi days post infiltration
DC3000 Pseudomonas syringae pathovar tomato DC3000
COR coronatine
CKs cytokinins
EAR ERF-associated amphiphilic repression
ET ethylene
ETI effector triggered immunity
ETS effector triggered susceptibility
FP forward primer
GA gibberellins
hpi hours post infection
HR hypersensitive response
hrp hypersensitive response and pathogenesis
HUB37 - AT5G47390
JA jasmonic acid
JA-Ile jasmonic isoleucine
JAZ jasmonate – ZIM domain
KO knock-out
L0 level zero
L1 level one
L2 level two
MAMP microbial-associated molecular pattern
NINJA novel interactor of JAZ
PAMPs pathogen associated molecular patterns
PCR polymerase chain reaction
P1 position one
P2 position two
PRR pattern recognition receptor
PTI PAMP triggered immunity
RD repressor domain
RP reverse primer
SA salicylic acid
TFs transcription factors
TR transcriptional repressor
TPL topless
TU transcriptional unit
T3SS type III-secretion system
ZIM zinc finger protein expressed in inflorescence meristem
1. Introduction

Aims and objectives

Hypothesis

Only very recently has it been recognised that transcriptional repression and the removal of negative regulators provide the capacity for a rapid and robust response to stresses. Here, two lines of research were undertaken to examine the roles of two EAR domain containing repressors in the defence responses of Arabidopsis thaliana to the hemibiotrophic pathogen, P. syringae pv. tomato strain DC3000. HUB37 is a MYB transcription factor that directly binds DNA. JAZ5 contains two EAR domains and targets transcription factors involved in jasmonate signalling.

AT5G47390 (HUB37) - is hypothesised to be a target of DC3000 effector proteins and/or part of effector-triggered immunity based on transcript modelling. Knockout lines of HUB37 are more resistant to DC3000. The protein level of HUB37 is yet to be quantified in response to infection with virulent and non-virulent DC3000. HUB37 has a conserved EAR domain. It is believed that the EAR motif is the active repressive domain of HUB37 interacting with other transcription factors. It is hypothesised that EAR mutated lines are therefore expected to act like hub37.

AT1G17380 (JAZ5) – has conserved domains which include two EAR domains. It is hypothesised that these two EAR domains contribute to JAZ5’s function as a dominant transcriptional repressor. This study seeks to determine the role of these JAZ5 EAR domains in plant immunity and JAZ5-JAZ10 interactions through generating targeted mutations in the JAZ5 EAR domains.

To enable molecular dissection of the role of EAR domains, constructs were generated to mutate EAR domains and epitope tag wild type and mutated JAŻ/HUB37 lines to characterise the role of these TRs in regulating plant immunity.
The contribution made by the thesis in the context of the approved field of study

This thesis addresses a neglected area of plant-pathogen interactions, that is, the role of EAR domains in regulation of plant defence. Overall this research will help increase our knowledge of jasmonate signalling and illustrate the utility of systems biology approaches.

It will generate new resources including *A. thaliana* JAZ5 with a combination of EAR domains mutated with epitope tags. This can be used to identify *in planta* expression and screen yeast 2 hybrid libraries.

Finally, it will characterise a novel MYB transcriptional repressor, which to date has not been implicated in plant defence responses.
Literature Review and Background Studies Underpinning this Study.

Plants are the source of organic carbon to almost all non-photosynthetic organisms on earth. They are sessile organisms but live in complex environments and agricultural productivity is affected by biotic and abiotic interactions. Plants have evolved sophisticated mechanisms to respond to microbe attack. The ability of pathogens to overcome the defence mechanisms of plants usually involves rapid evolution via natural selection for beneficial mutations within the pathogen population. As phytopathogens reproduce rapidly they can evolve to overcome plant immunity, despite concerted breeding efforts and often cause massive crop losses (Agrios, 2005). Such crop losses are exacerbated in many areas where elite varieties are monocultures of genetically identical plants and are hence susceptible to attack by pathogens and bacteria that have rapidly evolved to overcome the host resistance (Smith et al., 2010). Climate change, along with an increased demand on resources from a growing population, requires a greater understanding of plant microbe interactions. Today, crop losses through disease is one of the most significant factors impacting food security in both developing and developed countries. Population increase, ~ 9 billion by 2050, means that higher crop yields are required. Significant investment is required to develop new and innovative approaches to improving crops while limiting agrochemical use (Tomlinson, 2013). Regular spraying of crops with chemicals brings both financial and environmental concerns. If farmers are to be successful, they need access to disease resistant crops and have the appropriate biological and chemical measures to protect them.

Plants are normally resistant to most microbes. This resistance may be complete but can vary from partial immunity to complete susceptibility. Physical barriers like surface waxes and pattern recognition receptors on the plant cell surface provide the first level of immune protection. The latter recognise specific conserved molecules on the microorganism and this causes the activation of plant defences known as the basal defence mechanism. These defences are also known as pathogen-associated molecular patterns (PAMPs) or PAMP-
triggered immunity (PTI; Janeway, 1989; Dangl & Jones, 2006). Ideally, PTI operate at the point of entry and will prevent ingress, reproduction and spread of the disease throughout the plant. Localisation of the pathogen is imperative in the resistance of a plant to disease (Agrios, 2005).

Pathogenicity genes and disease specific genes are essential for a pathogen to infect a plant (Mansfield et al., 2012). These genes include those which: allow a pathogen to recognise its host; attachment of a pathogen to the surface of a plant; production of infection structures on the surface; invasion of the host (suppression of immune systems); production of toxins, and the capacity to reconfigure plant metabolism for nutrition and reproduction. Therefore, these virulence genes make a microorganism capable of causing disease and when their function is impaired, there is a loss or reduction in symptoms (Agrios, 2005; Boehm et al., 2014).

**Plant microbe interactions**
When a pathogen successfully infects a crop, for example *Phytophthora infestans* commonly known as potato late blight, it can lead to total crop loss and famine (Irish potato famine 1845-1848). We need detailed knowledge on systemic and localised defence to provide agricultural opportunities to increase crop yield by developing pathogen and drought resistant plants, minimising losses from seed to consumption (*Plant cell*, 2011).
Plants and pathogens have coevolved resulting in complex layers of plant defence mechanisms. Plants have elaborate signalling networks to defend against pathogens (Durrant et al., 2004; Pozo, Van and Pieterse, 2004). In turn plant pathogens have developed innovative strategies to modify plant signalling networks by invoking an array of counter tactics. These include hijacking, evading, disrupting hormone signalling pathways and/or crosstalk which is achieved through pathogen-derived hormones known as effectors. These effectors (virulence factors) target plant receptors, transcriptional activators/repressors along with other components to enhance microbial fitness (Vidaver and Lambrecht, 2004).

Plants have an innate immune system whereby transmembrane pattern recognition receptors (PRRs), such as receptor-like kinases, recognise
microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs), such as flagellin, invoking pathogen triggered immunity (PTI) (Zipfel et al., 2004). After potentially evolutionarily unavoidable PRR detection, the pathogen releases effectors to mask PTI by interfering with PAMP and/or subsequent defence signalling. For example, the type III secretion system (T3SS) is essential for hemibiotrophic pathogens such as P. syringae to deliver effectors. Successful reprogramming of the plants transcriptome, proteome and genome is known as effector-triggered susceptibility (ETS). See Figure 1.1, ‘A Simplified View of Plant-Pathogen Interactions’ from Kazan and Lyons, (2014). However, plants have coevolved an arsenal of resistant (R) genes encoding intracellular proteins with nucleotide binding domains, but this is not a defining characteristic. Successful R-mediated defence from the plant is known as effector triggered immunity (ETI).

Interestingly, strong necrotrophic pathogens which release powerful non-specific toxins and defence suppressing enzymes can also override PTI and ETI processes.

**Phytohormones**

Plant hormones are critical for defence against biotic and abiotic stressors. Phytohormones are chemical messengers that coordinate cellular activities. The key primary phytohormones include: jasmonates (JAs), salicylates (SAs) and ethylene (ET) which are well established. Other phytohormones e.g., abscisic acid (ABA), auxins (indole-3-acetic acid [IAA]), cytokinins (CKs), brassinosteriods (BRs), gibberellins (GA) and strigolactones work alone or in conjunction with primary phytohormones, seen in Figure 1.2 (Kazan and Lyons, 2014; Robert-Seilaniantz et al., 2011; Williams, M., 2010; Torres-Vera et al., 2014). Some pathogens also produce hormones, but plant derived phytohormones are often synthesised through different biochemical pathways which suggest they evolved independently from one another (Robert-Seilaniantz et al., 2011; Kazan and Lyons, 2014). This study focuses on components of the jasmonate and ABA signalling pathways that our laboratory has shown to play a key role in suppressing plant defence (de Torres et al. 2007, deTorres et al., 2015, Lewis et al., 2015).
Pathogen Secreted Effectors and Toxins

Diagram and text from Kazan and Lyons 2014, Figure 2

Figure 1.2. Complex Signaling Interactions among Phytohormone Pathways Regulate Both Disease Resistance and Susceptibility in Plants in Attacker-Dependent Manner

The plant hormones Jasmonic Acid (JA), salicylic acid (SA), and ethylene (ET) are primarily involved in plant defence. ABA, auxins (IAA), cytokinin (CK), brassinosteroid (BR), gibberellin (GA), and strigolactones (STR) also regulate plant defence, either alone or in conjunction with the primary defence hormones. Pathogens have developed strategies via their effector repertoire to either interfere with or hijack phytohormone pathways to induce resistance or susceptibility. Forward and blunt arrows indicate positive and negative interactions, respectively.

Pathogen Virulence Strategies

Different host environments are required depending on the type or lifecycle phase of the pathogen i.e. biotrophic or necrotrophic. Necrotrophic pathogens will change the plant environment to promote cell death so it can gain nourishment from those cells. On the other hand, biotrophic cells will prevent
cell (host) death. This complicates host responses, a classical example being
that JA and SA pathways can act antagonistically depending on where the host
is responding to a biotroph or necrotrophy (review in Thaler, Humphrey and
Whiteman, 2012).

Successful microbial pathogens have developed a number of strategies
including the production of plant hormones, phytohormone mimics and effector
proteins to overcome plant defence. *Pseudomonas syringae* pv. *tomato*
DC3000 (DC3000) translocates approximately 28 virulence effector proteins
into plant cells via the T3SS (Collmer *et al*., 2000; Greenburg and Vinatzer
2003; Cunnac *et al*., 2009). It is highly virulent on *A. thaliana* due to the
virulence factors (effectors) delivered through T3SS as they collectively
suppress MAMP-triggered immunity and ETI. The suppression enables DC3000
to multiply in the host (ETS) (Ward *et al*., 2010; Macho & Zipfel, 2015). By
contrast, DC3000*hrpA*- is mutated in an important structural component of the
pilus and prevents the T3SS from functioning (Roine *et al*., 1997). DC3000*hrpA-
triggers MAMP-triggered immunity and can’t secrete effectors. This significantly
lowers the virulence to *A. thaliana*.

Effectors are often defined as a “low molecular weight and cysteine-rich protein
secreted by pathogens during their interaction with plants and thus are both
proteinaceous and non-proteinaceous (e.g. toxins and nucleic acids)” (Kazan
and Lyons, 2014). DC3000 can also produce and secrete phytotoxins (Bender
*et al*., 1999). Phytotoxins are not always required for pathogenicity, but they do
enhance pathogen virulence in host plants (Bender *et al*., 1999). For example,
DC3000 produces coronatine (COR), which is a polyketide toxin. This activates
the JA signalling pathway through mimicry of jasmonoyl-L-isoleucine (JA-Ile)
(Verhage, van Wees, and Pieterse, 2004). COR is required for full virulence in
*A. thaliana* and tomato plants (Brooks *et al*., 2004; Ma *et al*., 1991; Mittal and
Davis, 1995; Zhao *et al*., 2003).

A common consequence of effector proteins activity is to manipulate the plant
phytohormone pathways, as previously mentioned (Schenk *et al*., 2000; Grant
reviews 2009 and 2011). By altering such pathways, the pathogen can alter the
host’s developmental and/or physiological features including stomatal opening
which would allow further infection opportunities (Kazan, 2014). In addition, pathogens cunningly take advantage of intricate crosstalk between phytohormones (Figure 1.2). In this way the pathogen not only changes one phytohormone but also impacts on an array of phytohormones; which can alter both resistance and susceptibility. The pathogen can suppress one hormone to promote another or vice versa, which ultimately leads to plant susceptibility.

**Transcription Factors**

Although research into transcription factors is growing, the function and target gene(s) of most transcription factors remain to be characterised (Pruneda-Paz et al., 2014). Understanding such responses is required to improve agricultural yields (Joshi et al., 2016). Transcriptional repressors can contain several domains, for example, DNA-binding domains and repressor domains. Repressor domains, such as ERF-associated amphiphilic repression (EAR) motif – the focus of this thesis - is conserved across many plant species, see Figure 1.3 from Kagale and Rozwadowsli (2010). However, its function and cellular fate(s) is currently not well understood, particularly in plant disease and defence. It is likely that the EAR motif has potential novel roles in plant-pathogen interaction and processes other than just transcriptional repression.

The regulation of gene expression is crucial for ensuring developmental programmes and response to environmental stress. Such response includes energy management, organisational maintenance, generating phenotypic variance and response to environmental stress.

Regulatory proteins known as transcription factors (TFs) underpin the first steps of gene expression. TFs individually or collectively instruct which genes to transcribe, how much, when and where. TFs can be activators or repressors and are characterised by their domains and interaction with other TF proteins, known as co-regulation. TF can not only control other TFs but it can alter expression of itself by positive or negative feedback. Furthermore, a TF may act as an activator or repressor depending on the cellular environment (Ikeda et al., 2009).
Diagram and description from Kagale and Rozwadowski (2010), Figure 1

“Arabidopsis EAR motif-containing proteins described in the literature. The 49 proteins are divided into two groups based on the sequence conservation pattern within the core EAR motif sites (highlighted in color). The alignment includes 12 amino acid residues upstream and downstream of the EAR motif, or up to where the nominal 12-amino acid sequence is abridged by encountering the first or last amino acid of the protein. A, The DLNxxP motif is conserved in some members of class II ERFs, TFIIIA-type ZFPs, and ABI3/VP1 family proteins. B, The LxLxL motif is conserved in AUX/IAAs and some members of the MYB and HD-Zip family proteins. Sequence logos (Crooks et al., 2004) illustrating the frequency of amino acids within the EAR motifs are presented below the respective alignments.”

Figure 1.3 A. thaliana EAR motif
Kagale and Rozwadowski (2010) identified DLNxxP motif and LxLxL motif containing transcriptional regulators in A. thaliana.
TFs can interact with a specific cis-regulatory DNA sequence and other proteins forming a transcriptional complex, see Figure 1.4 (Alberts et al., 2007). Distinct cis-regulatory modules provide the temporal and/or spatial component for the entire gene regulatory region. However, it is important to note that multiple cis-regulatory modules on the same regulatory region are more likely to act together rather than as a single universal promoter element (Benfey and Chua, 1990; Benfey et al., 1990; Davuluri et al., 2003). When mutating TFs with multiple cis-regulatory regions it is important to construct individual and collective combinations. It has been estimated that up to ten percent of plant genes encode TFs, which is twice the amount relative to animal genomes (Kaul et al., 2000; Riechmann et al., 2000; Mitsuda and Ohme-Takagi, 2009; Pruneda-Paz et al., 2014). This evidence suggests that TFs in plants have a greater potential to regulate and fine tune gene expression.
There is a gap of knowledge between TFs structural properties and their function (Hiratsu et al., 2003). Indeed, determining which TFs are capable of performing protein-protein and protein DNA interactions in in vitro studies is one of the major challenges. Furthermore, in vivo functional interactions that regulate genes through post transcriptional regulation and TF specificity adds to this complexity. TFs specificity varies; some recognise a broad spectrum of DNA sequences, yet others are more specific. TFs conserved motifs can denote their TF families that are more normally related to function. For example, JAZ proteins have the highly conserved Jas motif that mediates JAZ degradation. Nonetheless, different TF family motifs may overlap making experimental interpretation challenging and this is an area of intense research.
Transcriptional Repressors

A key pathogen virulence strategy is to alter host transcription to promote disease (Lewis et al., 2015). TFs can act as switches (on and off) in regulatory cascades (Smith et al., 2010) and the ability of pathogens to manipulate or hijack these provides a mechanism to suppress host immunity. One powerful mechanism to rapidly alter a transcriptional immune response would be to remove a transcriptional repressor. One important transcriptional repressor motif which has been identified is the ERF-associated Amphiphilic Repression (EAR) domains (Kagale & Rozwadowski, 2010).

Kagale and Rozwadowski acknowledged “in recent years, transcriptional repressors have emerged as important elements essential for establishing intricate spatial-temporal patterns of gene expression during plant development and plant responses to stress and hormonal signals”. Transcriptional repressors can contain numerous domains, for example, DNA-binding domains and repressor domains. Repressor domains include the EAR motif which is conserved in plants. Other repressor domains were identified in 29 A. thaliana TFs which differ from EAR domains (Ikeda and Ohme-Takagi, 2009). As stated, it’s function and cellular fate(s) are currently not well understood (Kagale, Links and Rozwadowski, 2010). The EAR motif potentially plays a novel role in plant-pathogen interaction and processes other than transcriptional repression.

Critically, specific deletion or mutation of an EAR motif found in a TF can abolish the repressive function, therefore modifying the overall function of the TF.

This research focuses on EAR domains in plant immunity, exploring the role of two key transcriptional regulators, described in detail below – a novel MYB transcription factor of unknown function and a specific member of the jasmonate repressor family, JAZ5. Until this work, there was no evidence that HUB37 plays a role in plant pathogen interactions.
The ERF-associated amphiphilic repression (EAR) motif

The EAR motif is a plant-specific active repressor domain (RD). It was first identified in tobacco ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 3 (Ohta et al., 2000). The EAR domain is present in many TF associated with plant defence and stress functions (Kazan, 2006). The motif comprises of two small conserved patterns, LxLxL and DNLxxP, giving the amphiphilic feature composed of leucine, an acidic amino acid (Hiratsu et al., 2004), see Figure 1.3. The molecular mechanism of transcriptional repression via the EAR motif is yet to be clarified.

Proteins with EAR motif(s) negatively regulate genes involved in developmental, hormonal and stress signalling pathways that have key biological functions. When an EAR-motif associates with a transcriptional activator they function as a dominant repressor (Hiratsu et al., 2003). This could provide an influential biotechnological tool for human and plant gene expression therapies. Kagale, Links and Rozwadowski’s (2010) analysis suggests that the EAR motif is the most predominant form of transcriptional repression so far identified in plants.

It has been suggested that chromatin remodelling may be involved with the function because the EAR motif interacts with one of the most important generic transcriptional regulators, TOPLESS (TPL), and mutations in HISTONE ACETYLTRANSFERASE GNAT SUPERFAMILY 1 suppress the tpl-1 phenotype (Long et al., 2006; Szemenyei et al., 2008; Mitsuda and Ohme-Takagi, 2009), Notably, TPL and its related “Topless Like” transcription factors play an important role in plant immunity.

MYB Transcription Factors

Transcription factor families like MYB are present in both animals and plants, thus inferring a common unicellular ancestor which have expanded and evolved novel functions through evolution. MYB transcription factors are conserved in many eukaryotes implying evolutionary significance (Nagano, 2000). Recent publications of MYB in humans have linked it to several types of cancers (Grotewold, Chappell and Kellogg, 2015; Fry and Inoue, 2018). Plants have an especially high number of MYB TFs that have evolved unique properties
specific to plants (Richman et al., 2000; Pireyre et al., 2015). There are 160+ members in *A. thaliana* and 220+ in rice. Due to the plant-specific functions it was hypothesised in 1997 that MYBs are important for plant form and metabolic diversity (Martin and Paz-Ares, 1997). MYB proteins can be passive repressors or passive regulators and they can interact homo- and hetero-dimerize as well as interacting with other proteins.

The MYB TF family is divided into subclasses according to the structure of the DNA binding domain. It contains one, two or three repeats (R1, R2 and R3) consisting of approximately 53 amino acid residues giving rise to a helix-turn-helix structure. The N-terminal R2R3 domain makes direct contact to the major groove of DNA and is highly conserved within the whole family (Dubos et al., 2010). The two clear surfaces allow the TF to bind to the DNA and is available for protein-protein interaction at the same time. This can be described as ‘solvent-exposed’ for the protein binding site (Grotewold, Chappell and Kellogg, 2015). Current opinion suggests MYB and basic helix-loop-helix TFs evolved in parallel and are associated with developmental and metabolic plasticity (Feller et al., 2011). The observation that ectopic expression of a MYB-related TF (AtMYBL) that modulates ABA and salt stress response in *A. thaliana*, causes early leaf senescence and suggests that AtMYBL mediated leaf senescence is mediated by ABA (Zhang et al., 2010 and 2011).

There is an evolutionary relationship between rice and *A. thaliana* MYB proteins; MYBS3 plays an important role in both rice and barley seed germination by regulating the depletion of carbon reserves (Lu et al., 2002; Rubio-Somoza et al., 2006). In addition, MYBS3 is involved in the cold stress tolerance in rice (Su et al., 2010). The understanding of MYB TF has important implications in monitoring gene expression in various scientific areas.
Discovery of the MYB At5g47390 (HUB37) in plant defence

Recent high resolution transcriptomic data has provided unprecedented temporal resolution of the A. thaliana gene regulatory network responding to the phytopathogen P. syringae, see Figure 1.5.1. A microarray gene expression time-course experiments which covered 13 time points over 17.5 h following DC3000 infection identified 1005 TFs differentially expressed suggesting they were targeted by DC3000 effectors. Through detailed network modelling of the data a (then) novel MYB transcription factor At5g47390 (HUB37) was identified to play a significant role in a regulatory network; predicted to be a hub in ABA signalling (sup1. Figure 1). Interestingly, upon further analysis it was found that

Three totally different expression patterns upon infection

Figure 1.5.1, Data derived from the PRESTA consortium (see Lewis et al., 2015)

Transcriptomic data of A. thaliana gene regulatory network responding to P. syringae. Expression pattern of different A. thaliana lines over time post inoculation.
MYB TFs closely related to HUB37, HUB17 and HUB23, show significant transcriptional differences in response to DC3000 infection. Notably, HUB23 shows a similar expression to HUB37 but HUB17 has a remarkable contrasting expression patterns.

Lewis et al. (2015) reported statistically significant co-expression differences across two of their three conditions; Mock, DC3000hrpA- and DC3000 (see Figure 1.5.2). Here they tested for enrichment of TF binding motifs in gene promoter sequences. DC3000 reduced protein accumulation compared to non-virulent DC3000hrpA-.

**Figure 1.5.2. Time course expression data from Lewis et al., 2015 Figure 7**

“Wigwams modules containing genes showing statistically significant coexpression across at least two of the three conditions were tested for enrichment of TF binding motifs in gene promoter sequences. Genes containing enriched motifs in their promoters were identified. In all cases, the mean expression profile of representative genes is shown (green, mock; purple, DC3000hrpA-; red, DC3000) with shading indicating SD.

(A) Genes co-expressed during DC3000hrpA- and DC3000 infection and containing a MYB TF-binding motif (PLACE: S-000355) in their upstream 500-bp sequences.”
HUB37 is predicted to be a highly connected TF in the ABA immunity signalling network

Previous Bayesian State-Space modelling using high resolution microarray time-course data of bacterial infection (Lewis et al., 2015) of the ABA perception and signalling network identified HUB37 as a highly connected protein (Figure 1.6).

AT5G47390, HUB37 appears to be a central node in ABA perception and signalling network.
Interestingly contrary to nearly all the other genes in the network model \textit{HUB37} which encodes an EAR motif, is strongly suppressed during bacterial infection. Within this network \textit{HUB37} is predicted to interact with key regulators of ABA signalling and directly or indirectly interact with repressive nodes such as TOPLESS, TOPLESS Related 1 and NINJA (Pauwels, 2010; Kagale and Rozwadowski, 2011). TOPLESS proteins are central regulators of transcriptional complexes and well documented to be involved in mediating plant defence responses (Figure 1.7). NINJA is part of the ABI5 Binding Protein family, originally discovered for its role in ABA signalling but more recently

\begin{center}
\textbf{Diagram and description from Pauwels \textit{et al.}, 2010.}
\end{center}

\begin{figure}[h]
\begin{center}
\includegraphics[width=\textwidth]{diagram.png}
\end{center}
\caption{\textit{NINJA connects the co-repressor TOPLESS to jasmonate signalling. Model for a general function of TPL proteins in plant hormone signalling}}
\end{figure}

\begin{enumerate}
\item[a] In the absence of jasmonates, bHLH MYC factors interact with the Jas domain of JAZ proteins that interact through their TIFY motif with domain C of NINJA. The EAR motif of NINJA is essential for interaction with the TPL co-repressors. \item[b] In the presence of (+)-7-iso-JA-L-Ile, JAZ proteins interact with the ubiquitin ligase SCFCO11 leading to proteosomal JAZ degradation and subsequent release of the NINJA/TPL complex from the MYC factors and activation of jasmonate-responsive gene expression. \item[c] Jasmonate and auxin pathways are built on similar signalling modules. \item[d] NINJA interacts with other group-II TIFY proteins which might be recruited by yet unknown transcription factors. \item[e] Interaction of the NINJA-related AFP proteins with ABI5 and TPL to regulate ABA responses.
\end{enumerate}
demonstrated to be involved in JA signalling, thus providing a possible mechanism for cross-talk between ABA and JA signalling (Figure 1.7). Thus, from this modelling data it was predicted that the EAR motif could be directly interacting with, and regulating other core genes in ABA signalling, and possibly be involved in JA-ABA crosstalk mediated by co-repressors.

A knockout (GK_783B02, knockout line N65033, stock name: CS365026) of At5g47390 was found to be more resistant to DC3000, see Figure 1.8 [M de Torres unpublished] and this finding was validated (as reported below).

Publications in 2013, 2014 and 2015 indicates At5g47390 has a role in a range of diverse plant processes, but to date has not been implicated in plant defence. The first paper, published by Yermin Kwon 2013, named At5g47390 ‘MYBH’. Over-expression of MYBH caused hypocotyl elongation by enhancing auxin accumulation. This suggested that MYBH is involved in the positive regulation of dark-induced hypocotyl elongation. mybh, a T-DNA insertion knockout mutant (GK-783B02: NASC ID N365026) had no major phenotypic difference to the wild type Col-0, though we see slightly reduced growth (Figure 1.8). The
mutant, overexpressing line, MYBH, increased phytochrome-interacting factor accumulation and thus auxin biosynthesis. MYBH phenotypically had darker, curled leaves and increased secondary root number compared to Col-0. MYBH was shown to localise in the nucleus through GFP tagging (Kwon et al., 2013). This is consistent with MYB-like transcription factors. At5g47390 contains a motif R/KLFGV. Gibberellin biosynthesis inhibitor, paclobutrazol, blocked overexpression of At5g47390 and increased hypocotyl elongation. In summary, Kwon showed the MYBH promoter activates in the dark and MYBH transcripts accumulate in the dark.

In a 2014 publication, Dandan Lu identified At5g47390 as ‘KUODA1’, showing that it was involved in cell expansion, leaf development and final organ size by controlling the expression of peroxidases. The paper reported that At5g47390 is involved in circadian regulation and directly represses genes encoding for peroxidases that control reactive oxygen species homeostasis in the apoplast.

**Jasmonic Acid Signalling**

The plant immune system relies on complex hormone signalling networks. Jasmonic acid (JA) is required to adapt to biotic and abiotic stressors. JA is an oxylipin synthesised from the polyunsaturated fatty acid – linolenic acid. It modulates many physiological and developmental agricultural traits such as root growth, survival and fertility (Wasternack and Hause, 2013). It is involved in pollen maturation, growth inhibition and wound induced defence against biotic attacks (Park, 2002; Robson et al., 2010). JA inhibits growth processes and is active in reproductive development, pathogen resistance and senescence. JA is an important signal in wounding and pathogen attack which increases both at the site of infection and systemically. JA and salicylic acid (SA) play a central role in defence and have an antagonistic relationship (Glazebrook, 2005; Grant and Lamb, 2006; Gimenez-Ibanez and Solano, 2013). They orchestrate complex transcriptional reprogramming depending on the microbe attack. Necrotrophs are more sensitive to JA-defence and biotrophs are more sensitive to SA-defence. JA represses growth and promotes pollen maturation. It is well
established that the JA-dependent defence is crucial in host resistance to *Botrytis cinerea* (Penninckx *et al*., 1996; Thomma *et al*., 1998).

JA and SA mutually antagonise each other via phytohormone crosstalk (Robert-Seilaniantz, Grant and Jones, 2011). The SA pathway confers resistance to biotrophic pathogens. However, activation of this (SA) pathway suppresses JA signalling thereby compromising resistance to necrotrophic pathogens. On the other hand, activation of the JA pathway enhances resistance to some necrotrophic pathogens (*Botrytis cinerea*) but inhibits SA pathway and resistance to biotrophic pathogens (reviewed in Thaler *et al*., 2012).

Notably, many strains of *P. syringae* produce the phytotoxin coronatine which is actually a mimic of the bioactive jasmonate JA-isoleucine which binds to the COI1 receptor and targets JAZ proteins for degradation (Geng *et al*., 2014). Coronatine can hijack JA signalling to suppress plant immunity (Robert-Seilaniantz, Grant and Jones, 2011). It is remarkable that a pathogen has evolved a novel small molecule to modulate a key plant hormone signalling pathway.

JAZ proteins directly bind to MYC2 leaving it in a transcriptionally inactive state. In the presence of JA-Ile or COR, JAZs are ubiquitinated by the E3 ubiquitin ligase complex (SCF^{COI1}) and degraded by the 26S proteasome. This causes the release of MYC2 which can then function as a transcriptional activator of JA. This system has a negative feedback loop where by the production of JA represses JA signalling (Lorenzo *et al*., 2004; Melotto *et al*., 2006; Chini *et al*., 2007; Thines *et al*., 2007; Fonseca *et al*., 2009; Zhang *et al*., 2015). JAZs contribute to early basal and secondary plant defence responses. It has been shown that JAZs can cooperate. For example, JAZ5 and JAZ10 specifically cooperate to restrict COR cytotoxicity and pathogen growth through complex transcriptional reprogramming (de Torres *et al*., 2015; de Torres *et al*., 2016). The *jaz5/10* mutant has a rapid suppression of JA-related components upon bacterial infection compared to other wild-type and other JAZ combinations (Figure 1.9) (de Torres *et al*., 2015).
Four out of twelve JAZ members of the JAZ family contain EAR motif(s). JAZ5 contains both LxLxL and DLNxxP in the C-terminal and middle region respectively. The repressor activity of the EAR motif found in JAZ proteins is currently unknown. It is hypothesised that the EAR domain in JAZ proteins is responsible for their dominant repressive function, as found in AUX/IAA.

Contrary to previous understanding, transcription factors are not all activators and when considering transcriptional networks repressors need to be factored into network analyses. The aim of this project was to produce the tools to allow future analysis of the role of repressive EAR domains in plant-pathogen interactions, building on previous research that had implicated two EAR domain
containing proteins as playing a critical role as targets of immune suppression by the bacterial phytopathogen *P. syringae* pv. *tomato* DC3000. It focusses on a novel MYB transcription factor HUB37, predicted to play a central role in the *A. thaliana*-DC3000 transcriptional infection regulatory network. HUB37 contains an EAR domain that has the potential to act as a transcriptional repressor domain. This motif is shared by JAZ5, another transcription factor identified within our laboratory as playing a key node within the jasmonate branch of plant immune suppression, functioning in conjunction with JAZ10 (de Torres *et al.*, 2015). JAZ5 contains two EAR domains and we hypothesise it functions differently from HUB37 as JAZ5 does not bind directly to DNA.

Thus two distinct EAR domain containing proteins are, HUB37, that is predicted to have a central role in the immune ABA signalling network and, JAZ5, that functions with JAZ10 to protect the host from pathogen hijacking of jasmonate signalling and are the focus of this investigation. The majority of this project was spent developing tools, transgenic lines with epitope tagged HUB37 and JAZ5 with mutations in the respective EAR domain(s). The HUB37 lines were sufficiently advanced to enable initial characterisation of this MYB TFs role in plant immunity.
MATERIALS AND METHODS

Growth conditions

*Arabidopsis thaliana* seeds were sown in F2 compost (Levingthon’s, UK) and were vernalised for 2 days at 4 °C in the dark. After vernalisation, seeds were transferred to a short day growth chamber (10 h light, 100-125μEinstein/m²/sec at 22 °C day, 20 °C night). Individual seedlings were pricked after 10 days in P24 celled (5x5 cm) plastic inserts filled with a ratio of 3:1 F2 compost to vermiculite (Willian Sinclair Horticulture Ltd) and placed directly into watering trays. Trays were covered with a propagator lid to maintain high humidity for a further 4 days. Plants were grown for 4-5 weeks before experimentation (de Torres Zabala *et al.*, 2003).

Seed Selection

Seed Sterilisation

Seeds were surface sterilised using chlorine gas, by placing in an open microcentrifuge tubes were placed into a desiccator jar with a 250 ml beaker containing 100 ml of bleach in the centre. A total of 3 ml of HCl was added to the bleach and the desiccator immediately sealed and left to stand for 3-16 h (depending on number of samples) in a fume hood. Chlorine gas was released into the fume hood and tubes were left in the fume hood for 5 min to allow evaporation of chlorine gas then left open in laminar hood until the chlorine smell was absent.

Antibiotic Selection

Approximately 100 sterilised seeds were sown on Murashige and Skoog growth media (MS) plates with appropriate selective antibiotic; stratified at 4 °C for 2 days in the dark and then incubated at 22 °C ± 1 °C on a 12 h light 12 h dark cycle (short day) ~100μMol/m²/s, for 14-16 days (corresponding to a minimum of four true leaves). Individual seedling were then pricked to soil as described above.

Herbicide (BASTA) Selection

Approximately 0.5 ml of seed was evenly distributed across trays (260 mm x 310 mm) filled with F2 Levingtons compost. Seeds were stratified at 4 °C for 2 days in the dark and then incubated at 22 °C ± 1 °C on a 12 h light 12 h dark cycle (short day) with seed propagator for 10 days. Plants were then treated through root absorption with BASTA.
Basta® (1:900), active constituent 150g/L (13.52% w/w) glufosinate-ammonium]. Plants were observed for the presence of visible phenotypes. Basta-resistant plants were transferred to pots and grown to maturity.

All seed for homozygous selection were taken to at least third generation.

**Pathogen Material**

*Pseudomonas syringae pv. tomato DC3000 (Pst DC3000)* strain containing the empty plasmid pVSP61 was maintained on solidified King’s B (KB; King et al., 1954) media with antibiotic selection, rifampicin 50μg ml⁻¹ and kanamycin 25 μg ml⁻¹. Culture maintenance, preparation and plant inoculation described by Katagira and de Torres-Zabala (Katagiri et al., 2002; de Torres-Zabala et al., 2006; de Torres, Sanchez, Fernandez_Delmond, and Grant 2003).

**In planta Pst DC3000 inoculation**

*Pst* DC3000 bacteria were grown overnight in 10 ml liquid KB (King et al., 1954) media containing selective antibiotic at 28 °C. Overnight cultures were washed and resuspended in 10 mM MgCl₂ to OD₆₀₀ 0.2 ( ~1 x 10⁸ CFU ml⁻¹). Depending on inoculation strategy, dilution series were performed.

**Syringe Injection:** For bacterial growth curves the DC3000 cell density was adjusted to OD₆₀₀ 0.0002 (~1 x 10⁵ CFU ml⁻¹) (de Torres-Zabala et al., 2007).

Undamaged and fully expanded plant leaves were selected, typically three or four per plant. Each side of the central vascular vein on the abaxial surface was nicked with a razor blade and infiltrated using a 1 ml blunt syringe. Excess bacterial solution on the surface of the leaf was gently removed with a paper towel (Katagiri et al., 2002; de Torres-Zabala, 2003).

This method bypasses the effect of stomata closure on the amount of bacteria entering the apoplast. Physiological differences between mutants do not affect the route of entry. CFU and disease symptoms accurately represent plant defence to DC3000.
Spray inoculation: DC3000 cell density was adjusted to OD$_{600}$ 0.02 ($\approx 1 \times 10^7$ cfu ml$^{-1}$) in 10mM MgCl$_2$ and 0.02 % surfactant (Silwet) solution. Plants were well-watered the day before to ensure stomata are open. Rosettes were sprayed with bacterial suspension using a spray bottle then placed into a clear bag for 24 h to maintain high humidity, ensuring maximal open stomata (ensure the plastic does not touch the leaf of any plant) (Katagiri et al., 2002).

Plants were kept under normal growth conditions described above during infection. Ecotype Col-0 was used as the wild type control in all experiments (de Torres Zabala et al., 2006; Katagiri et al., 2002).

Population counts and data analysis

Plant leaves were infiltrated (OD$_{600}$ 0.0002; six replicates per genotype) and challenge plants grown for three days. Using a flamed sterile disc borer (number 2, 5mm diameter), one disk from each leaf*, with a total of 3 discs per plant were pooled into a 2ml microcentrifuge, containing 1ml of 10mM MgCl$_2$ and a metallic ~5mm ball. Samples were homogenised using a tissue lyser (Qiagen, West Sussex UK) for 2 min at 25 Hz.

For each sample a 10x serial dilution was performed to $10^{-3}$. For each dilution, 6 x 10 μl aliquots were plated on KB media Petri dishes (King et al., 1954) and left to dry under a Bunsen burner flame. Plates were incubated at 28 °C for approximately two days; until bacterial colonies were visible. Bacterial colonies were counted at an appropriate dilution (25-75 CFU/aliquots) under a light microscope. From the six replicates, the average bacterial count and the standard deviation was plotted. Significant differences were determined by Students t-test.

Statistics methods

All bacterial growth measurements were determined from five independent replicates, each comprising three challenged leaves per plant. Significant growth differences between treatments were determined by the Students t-test (P < 0.5), error bars representing the standard deviation (SD) of the mean. Experiments were repeated three times.
Western immunoblotting

Total protein extraction
Plant material was placed in a 2ml microfuge tube and snap frozen in liquid nitrogen, and 350μl of extraction buffer (100mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.2 % Triton 100-X, 2.5 mM DDT, 1 mM PMSF and protease inhibitor cocktail, Sigma) was added to each leaf sample. Using a single metallic ball (5mm tungsten, Qiagen), samples were ground using a tissue lyser at 25 Hz for 2 minutes. The mixture was centrifuged at 12 000 g (max speed) at 4 °C for 10 min.

Protein quantification
The protein concentration for each sample was determined using Bio-Rad protein assay (Bradford, 1976) and samples were equalised with the addition of extraction buffer.

SDS Page electrophoresis
Laemmli sample buffer (5×) was added and the samples were heated for 5 min at 95 °C. Samples were loaded and separated on 12 or 15% polyacrylamide gels depending on protein size (JAZ or MYB proteins). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 4 °C.

Western blotting and probing
The blotted membranes were blocked with 5 % semi-skimmed milk powder for 1 h at room temperature (RT) (18 °C) and probed with the following antibodies: anti-MYC rabbit (AbCam) and anti-HA RAT monoclonal antibody (3F10; Roche) both were used at 1:5000 and 1:10 000 dilutions, respectively, in TBST (Tris/HCl, pH 7.5, with 150mM NaCl and 0.1% Tween 20), for 1 h. Secondary horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-rat antibodies (Sigma-Aldrich), respectively, were applied at 1:20 000 for 1 h before developing the blots with X-ray film using an automated developer.

PCR Genotyping of mutants

Plant genomic DNA extraction
One young leaf was cut with sterile scissors and placed into a microcentrifuge tube. The leaf was crushed with a microcentrifuge pestle in 500 μl of Shorty buffer (0.2 M Tris-HCL-pH9, 0.4 M LiCl, 25 mM EDTA, 1 % SDS). To this 500 μl of phenol:chloroform was added and vortexed. Samples were then centrifuged at maximum speed (12 000
g) for 5 min at RT (18°C). To a clean centrifuge tube, 450 μl of the upper (aqueous) phase was pipetted into a centrifuge tube containing 450 μl of isopropanol and mixed by inversion to precipitate the DNA. Samples were centrifuged for 10 min at max speed (12 000 g) and supernatant decanted. Precipitated DNA pellets were washed with 200 μl of 70% ethanol, briefly vortexed, decanted and residue liquid removed after a quick spin using a pipette. The DNA pellet was re-suspended in 100 μl of sterile MQ water. The DNA was run on a low EEO agarose gel for quality control and then quantified to determine the DNA concentration.

**PCR Reactions**

PCR was performed with Taq Polymerase as follows: (Initial denaturation: 95 °C for 3 min, 35 cycles: 95 °C for 30 sec, annealing X° C for 30 sec, extension 72 °C for X sec, end cycle and final extension 72 °C for 10 min).

X° - Dependent on specific primer combination annealing temperature, optimum established via a gradient PCR.

X - Dependent on final extension (1 kb/ min).

**Gel electrophoresis**

Samples were loaded on 0.8-1.2% agarose gel (low EEO, Melford) containing ethidium bromide (0.2 μg/μl). The percentage of gel was determined by expected amplicon size. Gels were run in a tank with 1x TAE (40mM Tris, 2.2mM Na2EDTA) buffer. Molecular weight marker, 1 kb DNA ladder (NEB), was used as a standard. The PCR products were visualised on a UV transilluminator and photographed.

**Construct generation**

Constructs for HUB37, JAZ5 and JAZ10 were designed with epitope tags.

*HUB37* was constructed in a traditional manner using Clontech’s C1 pCambia 1032 (10549bp). Golden Gate Cloning technique (Figure 2.1) was used for JAZ construction
as this method enables multiple constructs to be generated using a level system, discussed in detail below.

![Diagram of Golden Gate Assembly]

*Figure 2.1 Schematic diagram of Golden Gate Assembly*

Golden Gate Assembly is based on a modular level system. Using restriction enzymes cutting specific 4bp overhangs; transcriptional units (TU) are assembled using a variety of level zero (L0) vectors with complementary overhangs giving direction. A gene of interest or promoter, for example JAZ5, can be inserted into L0 vectors. L0 vectors allow a great deal of variation, for example different epitope tags can be swapped in and out. Up to seven TU can be inserted into a level two (L2) vector allowing multiple TU to be inserted into a plant at once. L2 vectors include selectable markers, for example Kanamycin resistance; herbicide resistance cassette can be created in a level one (L1) units.

Hub37 expressed under 35SCaMV* overexpression promoter and was introduced into both WT (Col_0) and At5g47390 T-DNA insertion KO mutant (hub37) for overexpression and complementation studies.

* 35SCaMV promoter drives ectopic expression therefore pathogen phenotypes derived from this line could be hard to extrapolate as the high expression level is likely to distort interactions i.e. unspecific protein-protein/DNA interactions.
A C-terminal hemagglutinin (HA)-tagged was fused to HUB37. This enables identification of HUB37 for in vivo Protein-DNA interactions through Chromatin Immunoprecipitation (ChIP)-Seq. The HA tag sequence can be found in supplementary data (Supplementary Data 3).

**Cloning strategy for C1-35S\textsubscript{pro}:MYB:3XHA-NOS**

Constructs and T0 seed were generated by de Torres Zabala (unpublished). This was cloned into a modified pCAMBIA C1 vector containing a C-terminal MYB.HA tag by vector Ncol (filled) BglII and 5' Scal and 3' BglII (which removed the stop codon).

1. PCR triple HA from pHB1-HA3 with:
   
   \[
   \text{3HAtag5'} + \text{BglII} – \text{NcoI: gcta gat ctC ATG GCA GGT TAC CCA TAC GAC (Phusion)}
   \]
   
   \[
   \text{3HAtag3'} + \text{PmlI and STOP codon, reverse complement: CT TCT CTA CGT TCC TCT tGA cAC GTg CAC CGGTGcACGtgTCaAGAGGAACGTAGAG (Phusion)}
   \]

2. Clone in C1 was cut with BglII + PmlI: C1-3HA
   
   PCR At5g47390 from cDNA with 5' primer Scal (AGT ACT blunt) + and 3' primer + BglII (removing STOP codon)

3. Clone in C1-3HA was cut with Ncol filled and BglII

**HUB37 ORF** (1098bp) was amplified using cDNA with primers introducing EcoRV sites, then cut with EcoRV and cloned in Cambia 1302 (C1) cut with Ncol filled and PmlI.

**Primers:**

At5g47390-START ggGATATCAGACTCGTGTGTTCTCCTAG;

At5g47390-STOP cctcagAtAtTTATAAGCGGTGTATCAGG.

**Yeast two-hybrid Assay (Y2H)** for protein-protein interaction against a general library of proteins.

Future work envisaged interactors would be confirmed by an in vivo pull down experiment, for example Tap-Tag to see in planta protein-protein interactions.
HUB37 Protein Degradation *in vitro* with DC3000

To test this, initial experiments were conducted that involved non-inoculated total leaf protein extracted from HYB37:HA tagged lines being mixed in a 1:1 ratio with DC3000 inoculated Col-0 leaf (non-transgenic) protein extract. Mixtures were incubated at room temperature (18 °C) for up to 18 h before SDS loading buffer was added to stop the reaction. It was hypothesised that HUB37 would degrade at a faster rate with Col-0 inoculated total protein extract compared to non-inoculated total protein extract due to the defence response. This was the first attempt to test if the abundance of HUB37 decreased over time *in vitro* as previously found *in planta*.

Golden Gate Assembly of *JAZ5, JAZ10* and *JAZ5 EAR* mutagenesis.

Golden Gate Assembly multiple constructs were constructed and transformed into plants using agrobacterium GV3101 as described by Holster, 1978. Molecular assembly strategy followed as described by Engler and Weber (Engler, Youles and Gruetzner, 2008; Engler *et al*., 2014; Weber *et al*., 2011). Please refer to Golden Gate Cloning strategy diagram for level assembly strategy (Figure 2.1).

JAZs contribute to early basal and subsequent secondary plant defence responses (de Torres, 2015). Detailed genetic analyses revealed that *JAZ5* and *JAZ10* function co-operatively compared to other JAZ proteins (de Torres Zabala, 2015). Their co-operative behaviour attenuates phytotoxicity mediated by the bacterial phytotoxin coronatine (COR) and to moderately restrict bacterial growth (de Torres Zabala, 2015). For this reason, *JAZ5* was proposed to be tested on its own and with *JAZ10* to establish if the *JAZ5* ear domains affected the co-operative behaviour.

Different N-terminal epitope tags were used for *JAZ5* and *JAZ10* to localise each protein *in planta*. It has been reported that *JAZ10* has four splice variant proteins (Chung *et al*., 2010) and for this reason an N-terminal tag was used for both *JAZ5* and *JAZ10*. The native promoters of ~1200bp were used to ensure usual hormone
responses and not to impact on the fine-tuned signalling network. This allowed direct comparison to WT and KO responses to the pathogen.

The native A. thaliana Actin2 terminator sequence (Act2 ter.) was used to terminate expression for JAZ constructs.

Positive colonies were selected through PCR amplification and digestion before being sequenced (Eurofins Genomics, Tube Sequencing).

**JAZ 10**

The JAZ10 promoter was amplified by High-Fidelity PCR (~1500bp), while primers with BpiI recognition overhang sites, and digested and ligated into L0 acceptor vector pAGM1251 with BpiI and T4 DNA ligase.

The JAZ10 Gene (1502bp) was amplified as above and ligated into pICH41808. 3x MYC (122bp) tag and Act2 ter. (485bp) were sourced from Golden Gate Modular Cloning Toolbox for Plants, pICSL30009 and pICH44300 respectively.

Following sequence validation, all L0 vectors were amplified in DH5a and linearised. A one-pot digestion and ligation reaction was performed to assemble the contigs into a Level one position 1 vector. After further selection, amplification and sequencing the JAZ10 cassette (7996bp) was ligated into a Level 2 vector along with a BASTA resistant cassette (11138bp) generating pGBKT7::JAZ10pro:MYC::JAZ10:Act2-BASTA.

The resulting construct was introduced to Agrobacterium competent cells (GV3101) using heat shock transformation method as described by Holster, 1978.

**JAZ 5**

Like JAZ10, the JAZ5 native promoter was amplified by High-fidelity PCR (~1500bp). The JAZ5 gene (~1400bp) was inserted into L0 acceptor vector pAGM1276, digested with BpiI and ligated in three sections. The Bsal restriction site was removed by Kit based mutagenesis in the L0 vector. Further mutagenesis was used to mutate one or both EAR domains using a series of appropriate primers (Figure 2.2). A different approach using pICH86966 was developed to assemble Level two Position two for JAZ5 WT, eari and eari/ii. In this approach, L0 constructs were ligated directly into pICH86966 with Kanamycin resistance, avoiding L1 construction. 3x HA (124bp) tag and Act2 ter. (485bp) were sourced from Golden Gate Modular Cloning Toolbox for Plants, pICSL30008 and pICH44300 respectively.
The resulting constructs were introduced to *Agrobacterium* competent cells (GV3101) using heat shock transformation method as described by Holster, 1978. Col-0, jaz5, jaz10 and jaz5/jaz10 plants were transformed with *Agrobacterium* containing the relevant construct by floral dipping.

\[
pGBKT7::JAZ5_{pro}:3xHA::JAZ5::Act2 - kanamycin
\]

\[
pGBKT7::JAZ5_{pro}:3xHA::JAZ5eari::Act2 - kanamycin
\]

\[
pGBKT7::BASTA-JAZ5_{pro}:3xHA::JAZ5earii::Act2.
\]

\[
pGBKT7::JAZ5_{pro}:3xHA::JAZ5eari/ii::Act2 - kanamycin
\]

---

**Figure 2.2 Overview of JAZ5 cloning and mutation strategy.**

*JAZ5* amplified in two fragments using primers to mutate the internal BpiI recognition site with BpiI overhangs to ligate two fragments back together on L0 vector. Using kit biased mutagenesis on this vector the internal BsaI site was removed. After verification, PCR amplification using eari mutated primer then and/or kit based mutagenesis of eari to mutate EAR domains. The kit was used to mutate eari as it is in the middle of the gene.
Plant Transformations
Transformations were performed with *Agrobacterium tumefaciens* strain GV3101 by the floral dip method (Clough and Bent, 1998).
Results

This results section covers the generation and testing of constructs that provide the foundation for understanding the role of EAR domains in plant pathogen interactions. As I initiated this project, the laboratory gained verification via *in planta* bacterial growth assay that HUB37 indeed had a biological role in suppression of plant defence due to increased resistance of a loss of function HUB37 allele following DC3000 challenge.

HUB37 Mutant Phenotype

One initial task was validating a biological role for HUB37 in plant defence. A T-DNA insertion mutant line (GK_783B02, knockout line N65033, stock name: CS365026) in *HUB37* (*At5g47390*) was generated and tested to be homozygous. This line was shown to be significantly more resistant to *P. syringae* DC3000 compared to WT Col_0, Figure 3.1.

---

**Figure 3.1.** *HUB37* is significantly more resistant than *Col_0*

A) Plants were inoculated with DC3000 0.0002 OD$_{600}$ and population counts undertaken 3dpi. Enumerating bacterial counts indicates that *hub37* is significantly more resistant than WT, students t-test (*P* < 0.05). B) *hub37* has smaller leaf size compared to WT.
HUB37 Selection Process

Transcription profiling showed a strong suppression of HUB37 expression during infection with DC3000. In order to determine how well the abundance of HUB37 protein mimicked the transcript levels during suppression of immunity, plants were transformed with the C1-35S\textsubscript{pro}:MYB:3XHA-NOS and homozygous lines selected.

The C1-35S\textsubscript{pro}:MYB:3XHA-NOS construct, was transformed into Col-0 or hub37 knockout lines and T0 seed generated by de Torres Zabala using standard protocols. Transformants from T\textsubscript{1} seed were selected on hygromycin MS plates, grown in soil, selfed and then the T\textsubscript{2} generation selected for homozygous lines on hygromycin MS plates, looking for 100% germination. Independent lines were then tested for HUB37 accumulation by Western blot of crude protein extracts with an antiHA antibody. Not all T\textsubscript{2} plants expressed MYB:HA, when tested through Western blot and plants positive on Western’s were taken to the T\textsubscript{3} generation (see Figure 3.2). Three independent lines for each construct were taken forward for further analysis. Phenotype of each line and levels of expression were selected in lines and a representative example of these are shown in Figure 3.3.
Figure 3.2. Accumulation of HUB37 in first generation (top) and third generation (bottom left, 4c5) transgenic lines.

Col-0 leaf phenotype larger than hub37. T₁ generation of transformed hub37 plants, with overexpression of HUB37:HA, appear to be larger than hub37 and similar to WT (Col-0). Col-0 with over expression of HUB37:HA do not appear larger, some in fact were smaller. Western Blot analysis, probing for anti-HA, indicated expression in the T₁ generation Col-0::CaMV:HUB37:HA (T₁ 4, T₂ C,T₃ 5). MYB:HA is degraded in the presence of DC3000 20hpi.
Figure 3.3 $T_3$ generation phenotype and levels of expression selected in lines
**HUB37 Accumulation Decreases Over Time When Plants are inoculated with DC3000**

Figure 3.2 suggests that HUB37 protein is not stable following DC3000 infection compared to DC3000hrpA, mock or unchallenged leaves. To investigate this further leaves from selected transgenic HUB37 lines were inoculated with *P. syringae pv. tomato* strain DC3000 (0.2 OD₆₀₀) and protein extracted over a time-course. Western blot with anti-HA antibody revealed that HUB37 protein appeared to degrade over time. Interestingly, over the time course, as the ~46Kd HUB37 disappears a smaller immunogenic protein band appears (Figure 3.4).

![Western blot](image)

**Figure 3.4. HUB37 protein decreases over time when plant tissue is inoculated with DC3000**

Western blot analysis showed HUB37 protein degrades (reduces) over time when plant tissue was challenged by DC3000. Leaves were inoculated with DC3000 0.2 OD₆₀₀ and snap frozen at 0, 8 and 20hpi. Proteins were extracted, normalised and run in a 10 % SDS-acrylamide gel. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane before being probed with hemagglutinin (HA) antibody for the HA tag on HUB37. Then a Horseradish peroxidase (HRP) secondary antibody enabled florescent imaging of HUB37 protein expression, depicted by the dark bands.
Virulent DC3000 caused HUB37 protein to diminish compared to non-virulent HrpA(-)

Figure 3.5 shows the level of HUB37 accumulation in 3 lines of one independent transformant (note plant 3 appears not to be transgenic). In line with previous microarray gene expression time-course data DC3000 reduced HUB37 accumulation compared to non-virulent DC3000hrpA (Figure 1.5.2) (Lewis et al., 2015), indicative of type III mediated degradation of HUB37. To try to quantitate the levels of HUB37, grey-scale scale analysis was used. In grey scale analysis quantifies brightness as a percentage, 0% is black compared to 100% being white; non-inoculated expression on average was 35%, Mock 49% (data had a comparatively lager range of 16%) DC3000hrpA 49% and DC3000 75%. Repeat experiments can be found in Supplementary Data 3.3.

Figure 3.5. HUB37 protein expression when challenged with DC3000 and hrpA
20 hpi time point of HUB37 abundance when challenged with DC300, DC3000hrpA or mock. Key: N - Non inoculated; M - Mock MgCl2; H – DC3000hrpA, OD_{600} 0.2; D – DC3000 0.2 OD_{600}. Using grey scale analysis, N - 35%, M - 49%, H - 49% and D - 75% indicating the brightness of black for each section.
HUB37 Protein Degrades at the same rate in vitro with DC3000

Due to the abundance of HUB37 decreasing over time during a DC3000 challenge it was hypothesised that it could be due to specific proteolytic activity activated by type III effectors. Abundance of other transcriptional repressors involved in plant defence, such as JAZ proteins, are controlled through ubiquination by the 26S proteasome (Thines et al., 2007). While this was only repeated once, Figure 3.6 shows that the abundance of HUB37 decreased over time in vitro as previously found in planta. Expression was not detectable in the Col-0 background lines (Supplementary Data 4.5). Future experiments can focus on optimising the method by altering the extraction buffer, temperature and increasing the concentrations of proteins (for Col-0 background) which may alter the enzymatic activity and provide higher sensitivity to help validate this exciting result.

Figure 3.6. in vitro DC3000 inoculation, HUB37 Degrades at the same rate

Degradation of HUB37 in vitro over time; Col-0 DC3000 inoculated protein extract was mixed in a one to one ratio with non-inoculated HA:HUB37 expressing lines. Clear degradation can be seen in the hub37 background over time. Challenged protein mix appeared to degrade more by 18 h compared to non-inoculated tissue. Panels show two independent experiments.
JAZ Cloning

Using Golden Gate cloning (see materials and methods section) JAZ5 and JAZ10 were expressed under their native promoters and epitope tagged in the N-terminus, as the C-terminus is implicated in key protein-protein interactions, and in JAZ10 alternative spliced C-terminal variants.

These provided the foundation for me to develop lines with JAZ5 EAR domains knocked out in various combinations, see Figure 3.7.

![Figure 3.7. Schematic diagram showing transcriptional unit variations for JAZ5 EAR domain mutation combinations.](image)

A combination of primers to amplify and mutate EAR domain i and ii in JAZ5. Golden Gate assembly transcriptional units (TU) could then be assembled with a choice of epitope tag and promoters.

JAZ10

After level zero (L0) vector assembly with the JAZ10 promoter it was sequenced from left and right vector borders across the whole inserted amplified promoter. This revealed an adenine insertion at ATG -1445 bp, see Figure 3.8. As this insertion was in the promoter with over 1kb distance from the gene, the process was not repeated and the vector was used later for Golden
Gate assembly. No mutations occurred across the full length of JAZ10 CDS which was sequenced in full (1502bp). Before proceeding to level two (L2), level 1 JAZ10 promoter, N terminal 4X MYC tag and JAZ10 gene junctions were sequenced which confirmed JAZ10 is in frame with the MYC tag, see Figure 3.9. Confirmation by PCR diagnostics and digestion showed position 1 MYC:JAZ10 cassette, position 2 BASTA cassette and end link 2 successfully ligated together in pGBKT7. The construct was successfully transformed into Agrobacterium competent cells GV3101 which were used for floral-dip transformation into Col-0, jaz5 and jaz5/10 plants. T₀ seed was harvested and positive transformants identified through BASTA selection.
At ATG-1445 an extra adenine inserted was found though sequencing indicated by the red box compared to the expected aligned sequence.
Figure 3.9  N-terminal MYC epitope tag in frame with JAZ10 gene.

The JAZ5 promoter and an N-terminal MYC epitope tag and gene successfully ligated together; JAZ5 gene was in frame with MYC tag.
Through Golden Gate Assembly and kit-based mutation using Quick-Change II site-directed mutagenesis kit (Stratagene), WT and mutated EAR domain(s) were constructed and expressed in WT (Col-0) and KO jaz5 and jaz5/10 double mutant lines (see Figure 4.0). All constructs were sequenced to confirm the corresponding mutation and assembly (see Supplementary Data 5 for primers and Figure 4.1 for amino acid changes).
For JAZ5, two internal restriction sites (BpiI and BsaI) first had to be removed to proceed with Golden Gate cloning. The “domestication” sites were positioned in the intron so the changes would not affect the final protein sequence. The BpiI restriction site was removed through PCR amplification (Figure 4.4) and digestion into level zero (L0) vector. Once in L0 vector, the BsaI restriction site was removed by Kit based mutagenesis (Figure 4.5). From this point, further mutagenesis using kit based mutagenesis was used to mutate both EAR domains. See Supplementary Data, 5 for a full primer list.

JAZ5 has two EAR motif domains (Figure 4.2 and Supplementary Data). The leucine residue corresponding to the EAR motif sequence, as described by Kagale 2010 (Figure 4.2), was altered to alanine; a basic, nonpolar amino acid (Kagale et al., 2010). This was carried out to diminish the repressive nature of the EAR motif and determine whether this altered JAZ5s interactions and function during plant pathogen interactions.
Level zero JAZ5 constructs were constructed in a similar way to JAZ10, with the exception that JAZ5 (1417bp) was amplified in two sections to mutate the internal BpiI site (using primers 31-34); JAZ5 pro (1557bp). Before proceeding to level one, an internal BsaI site was removed by site-directed mutagenesis (Stratagene) using primer 36 (Figure 4.4 & Supplementary Data section 5).

Once the mutation had been confirmed through restriction digestion and sequencing, the construct was used to proceed. eari was subsequently mutated by the same strategy using primer 1. Confirmation of eari (Figure 4.3) mutation was established through sequence alignment. Because earii is positioned at the end of the gene it was possible to mutate using primer 35 and high fidelity PCR. Using JAZ5 WT and JAZ5 eari as templates the second EAR motif was mutated using the earii primer, giving JAZ5 eari mutated and JAZ5 eari/ii mutated genes. These four JAZ5 constructs were then assembled into level one vector (see below), this time in position two, which later allowed double constructs to be assembled and sequence validated. The final constructs were:

pGBKT7::BASTA-JAZ5\textsubscript{pro}:3xHA:JAZ5:Act2

pGBKT7::BASTA-JAZ5\textsubscript{pro}:3xHA:JAZ5eari:Act2

pGBKT7::BASTA-JAZ5\textsubscript{pro}:3xHA:JAZ5earii:Act2

pGBKT7::BASTA-JAZ5\textsubscript{pro}:3xHA:JAZ5eari/ii:Act2

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<td>LDLRL</td>
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</table>

*Figure 4.2. JAZ5 conserved EAR motif position and sequence*

The location refers to the position of the Leu (L) residue underlined in the corresponding EAR motif sequence.

Figure adapted from Kagale 2010.
**Figure 4.3.** Sequence alignment of Level zero JAZ5 eari mutated constructs.

From sequence alignment JAZ5eari domain has been successfully mutated and is present in L0 plasmid. Stocks were made and stored for future use.
The JAZ5 gene was amplified in two sections. Primers over the internal BpiI recognition site cause a substitution removing the enzyme recognition sequence. The primer overhang contains a BpiI recognition site, so during L0 assembly using BpiI, fragment ends were digested and re-ligate together. Sequence alignment was confirmed with expected mutation.

HF-PRC amplified fragments mutated the internal BpiI recognition site. Fragments were digested with BpiI and ligated back together in a L0 vector. Sequencing across this section indicated mutation and ligation was successful.
Level zero (LO) JAZ5 gene WT, eari, and earii were assembled in level one (L1) acceptor vector, pICH4774 with the same stocks of JAZ5 promoter, Ac2 terminator and HA epitope tag. N terminal PCR diagnostic of positive colonies across the HA tag (JAZ5 promoter FP and JAZ5 gene RP) and gene-terminator junction (JAZ5 gene FP and Act2 RP) were amplified. From the six colonies of each assembly only JAZ5 earii mutated was positive. Repeat PCR testing of a larger number of colonies (twelve more) confirmed that only JAZ5earii could be taken forward (see Figure 4.6). Due to assembly miscarriage the other three were constructed directly into a level two-acceptor vector pICH86966 (see Figure 4.8). L1 position two (P2) JAZ5earii cassette was then successfully assembled into L2 acceptor vector with L1 P1 BASTA; pGBKT7::BASTA-JAZ5pro:3xHA::JAZ5earii:Act2. The construct was validated by PCR diagnostics, digestion and sequencing.

L2 pICH86966 - JAZpro:HA::JAZ5gene:Act_2

Due to unsuccessful level two, position two (L2P2) assembly described above, a different approach using pICH86966 was used. Appropriate L0 constructs were directly ligated into pICH86966, avoiding L1 construction using Bsal enzyme in a one pot digestion and ligation reaction (see Figures 4.7 and 4.8).
plCH86966::JAZ5<sub>pro</sub>:3xHA:JAZ5:Act2 - kanamycin

plCH86966::JAZ5<sub>pro</sub>:3xHA:JAZ5eari:Act2 - kanamycin

plCH86966::JAZ5<sub>pro</sub>:3xHA:JAZ5eari/ii:Act2 - kanamycin
A. L0 vectors with JAZ5 promoter, MYC tag, JAZ5 gene and Act2 terminator assembled in pICH86966.

Figure 4.7. L2 pICH86966 - JAZ5-HA-JAZ5-HAS assembly

Figure 4.7. Assembly of the L2 vector with the JAZ5 gene, MYC tag, and Act2 terminator.

Digestion using BsaI enzyme leaves specific 4bp overhangs to ensure the correct assembly order during ligation.

A. L0 vectors with JAZ5 promoter, MYC tag, JAZ5 gene and Act2 terminator assembled in pICH86966.
Figure 4.8. L2 pICH86966 - JAZ_{pro:HA}::JAZ_{gene:Act.2} assembly

B. L2 vector map, total vector size of 10,000 bp. L2 vector has a kanamycin resistance cassette with NOS promoter and terminator for selection.
Due to initial unsuccessful L2P2 assembly for JAZ5 WT, eari and eari/ii, a different approach using pICH86966 was developed. In this approach, L0 constructs can be ligated directly into pICH86966, avoiding L1 construction. A drawback to this approach is that the resistance cassette, already in the plasmid, is kanamycin. Kanamycin has a lower selection efficiency in plants compared to BASTA and can make the homozygous selection process longer.

Positive colonies selected through PCR diagnostics across the HA tag, Figure 4.9, and left border primer to promoter. Digestion of the plasmid confirmed the expected size. The resulted construct was introduced to Agrobacterium competent cells (GV3101) by heat shock transformation. Then later transformed into flowering plants by the floral dip method.

Col-0, jaz5, jaz10 and jaz5/jaz10 plants were transformed with Agrobacterium containing the relevant construct by floral dipping. Seeds were collected. Transformed seeds were then selected on kanamycin antibiotic agar plate or BASTA treated soil as appropriate.
Discussion

This project focusses on the role of transcriptional repression in plant immunity. Specifically it studies two *Arabidopsis thaliana* transcriptional repressors with diverse functions that have been experimentally validated to be targeted by bacterial effectors of the virulent hemi-biotrophic pathogen, *pseudomonas syringae pv. tomato* strain DC3000 (Glazebrook, 2005; Grant and Lamb, 2006; Robert-Seilaniantz, Grant and Jones, 2011; Gimenez-Ibanez and Solano, 2013).

Experimental evidence to date shows that these transcriptional repressors target two hormone pathways that are known to be modulated by DC3000 type III effectors, abscisic and jasmonate signalling (Greenberg and Vinatzer, 2003). One, HUB37, is a MYB transcription factor that has diverse functions being recently identified to have roles in hypocotol elongation (Kwon *et al.*, 2013; Lu *et al.*, 2014).

The other, JAZ5 is a transcriptional repressor of jasmonate signalling, functioning in conjunction with JAZ10 to attenuate bacterial virulence (de Torres *et al.*, 2015).

Both these immune signalling components have EAR domains, transcriptional repressor domains, notably, JAZ5 has two domains (see Figure 4.1) (Kagale, 2010).

This project set out to further characterise the function of JAZ5 and in particular HUB37 as its role in plant immunity has not been to date reported. HUB37, is so named as it was predicted to be a central HUB in a Bayesian State Space Model generated using gene expression profiles of DC3000 infected tissue over a 13 time point microarray experiment and modelled against known components of ABA-signalling and biosynthetic pathways (Lewis *et al.*, 2015; S. Jayaraman & M. Grant unpublished results). Strikingly HUB37 expression is strongly suppressed upon DC3000 infection, suggesting attenuation of HUB37 is necessary for full disease symptoms. Consistent with this, as I arrived in the laboratory, *hub37* KO line was shown to be more resistant to DC3000, though currently the mechanism behind this resistance remains to be elucidated. As
part of the initial work on the project I also looked at whether two other MYB genes differentially regulated between virulent DC3000 and non-pathogenic DC3000hrp and predicted to represent transcriptional hubs (HUB23 and HUB17) were functionally associated with HUB37. The hub37/hub17 double mutant showed additional enhanced resistance suggesting these two MYBs co-operated in promoting disease development (negative regulators of plant immunity). The core part of the initial project work involved characterised transgenic lines expressing HA epitope tagged HUB37 in both Col-0 and hub37 mutant backgrounds (transgenic primary transformants generated by Marta de Torres) under a strong 35S cauliflower mosaic virus promoter. This construct appeared to complement mutant hub37, but strikingly, I was able to demonstrate that HUB37-HA was degraded during disease development caused by DC3000 but not the DC3000hrp mutant (Figure 3.5). This is an exciting result as it is one of the few examples I am aware of, of a plant transcription factor being targeted for degradation by bacterial effectors early in the infection process. This result is somewhat counterintuitive, as the hub37 mutant is more resistant to DC3000 infection (Figure 1.6) (de Torres, unpublished). This suggests that HUB37 is a negative regulator of plant immunity but the data shows that it disappears during infection. The most consistent hypothesis to explain this, is that HUB37 negatively regulates either susceptibility genes or pathways required to promote pathogen virulence and it’s displacement early in the infection process may be mediated by a specific proteolytic degradation, hence the loss of the epitope tag. This was one avenue of research being pursued at the time I decided to write a Masters. Simultaneously, I had used GoldenGate cloning techniques to generate constructs of HUB37 with a mutated EAR domain and successfully cloned this variant under its own promoter into a T-DNA expression vector ready for transformation into the hub37 background.

In parallel to these studies I wanted to extend the novel studies of de Torres et al. (2015) who showed the JAZ5 and JAZ10 co-operated to confer immunity to DC3000 infection. The JAZ family of transcriptional repressors compromises 12 members and this was the first example showing co-operativity amongst the
JAZ proteins. Importantly, JAZ5 contained 2 EAR domains so I used GoldenGate cloning to generate JAZ5/JAZ10 constructs with both epitope TAGs and either individual, or both JAZ5 EAR domains mutated (Figure, 4.2)(Kagale et al., 2010). These were successfully generated and sequence validated and transformed into the jaz5/10 background just prior to completing my labwork. While disappointing to not take these resources forward into a PhD, it has been both extremely exciting and particularly gratifying to generate so many resources and, particularly, show HUB37 is a negative regulator of plant immunity and is targeted, either for specific proteolytic cleavage or non-specific degradation by effectors. There are a number of future experiments that are obvious to follow on the initial discoveries. One possibility to test in the future is the effect of a range of protease inhibitors on the stability of HUB37. Serine and cysteine protease inhibitors as well as inhibitors of the 26S protease were the initial ones being considered to test prior to prematurely completing this project (Bode, Halitschke and Kessler, 2013; Dielen et al., 2010).

In addition I wanted to address the nature of the protease activity as there are some parallels to the caspase cascades initiated during programmed cell death in C. elegans and humans (Conradt, Wu and Xue, 2016; Taylor, Cullen and Martin, 2008). To this end I predict that simple in vitro mixing experiments of cytoplasmic supernatants from transgenic lines expressing HUB37 with supernatant from DC3000 infected leaves 15 hpi could be a powerful method to develop, assay and dissect the protease activity using targeted fractionation coupled to mass spectrometry. These would be used to both identify cleavage substrates (or products of HUB37 – see above) and unbiased profiling for proteases.

While it has been frustrating to leave this work uncompleted, the resources developed are now being used at Warwick University. Specifically, a PhD student, Sara Abdelsayed is taking over this work, initially focussing on the HUB37 disappearance while at the same time generating homozygous lines from the JAZ constructs. One new avenue she is pursuing, instigated by my work, is cloning the hub37 promoter into a yeast 1 hybrid bait vector.
This will enable them to screen a transcription factor library to attempt to identify the transcription factors that are responsible for the observed suppression of the *HUB37* transcript following DC3000 infection.

Interestingly, another observation to test is a possible link between HUB37 and ABA signalling. Notably, in addition to be predicted as a key hub in ABA signalling the knockout line of *HUB37* was significantly more resistant to DC3000 than the WT Col_0 (Figure, 3.1), but also had a smaller phenotype compared to Col-0, strikingly reminiscent of the ABA biosynthetic mutant *aa03* which is also more resistant to DC3000 (de Torres *et al.*, 2009).

There are also some technical considerations going forward. The protein level of HUB37 determined in C1-35S*pro:MYB:3XHA-NOS* lines was quantified in response to infection with virulent and non-virulent DC3000. Notably western blot analysis of T₃ *MYB:HA* lines showed HUB37 accumulation was not consistent suggesting some lines were not stable after three generations of hygromycin selection and gene silencing may possibly be occurring (Figure 3.2).

Another issue to address with HUB37 (and relating to the above suggestion of looking at possible HUB37 cleavage products upon its disappearance) is that there is a clear ratio difference between protein size (46Kd) and a smaller immunogenic protein band (Figure 3.4). This indicates that as early as 8hpi there is a higher proportion of potentially degraded protein compared to 0hpi. To address this observation and characterise HUB37 integrity during early infection stages, the next step would be to analyse the 8hpi protein extraction using affinity pull down experiments (HA-affinity column). Through these experiments, it would be possible to establish the nature of HUB37 degradation during bacterial infection and identify potential cleavage sites by mass spectrometry.

In line with previous microarray gene expression time-course data DC3000 reduced HUB37 accumulation compared to non-virulent DC3000hrpA (*Lewis et al.*, 2015). This suggests the effector proteins secreted through the pilus of
DC3000 are the causal agent of both suppression of HUB37 transcript and directly HUB37 protein levels. Preliminary data (Figure 3.5) suggest effectors may not be the sole reason for this decrease in abundance of HUB37 as there also appears to be a small decrease in HUB37 abundance when inoculated with DC3000hrpA. However, this difference is minimal compared to mock and DC3000 which suggest protein expression is targeted directly by effector proteins and is involved in ETI. Further studies using more quantitative approaches, such as mass spectrometry as discussed above, are needed to determine whether PTI impacts HUB37 abundance.

The initial results indicate that the EAR mutated lines do act like hub37. These data support the hypothesis that the EAR motif is the active repressive domain of HUB37 interacting with other transcription factors. However, further analysis is obviously required. Using a pKUA1::GUS expression line to study leaf development it was shown that At5g47390 promoter activity was observed on day 12 at the leaf tip and is present throughout the leaf by day 14, but becomes restricted towards the base of the blade at day 17 (Lu et al., 2014). This is consistent with strong activity during the leaf expansion period and may account for the reduced stature of hub37 knockout lines.

The JAZ5 work is less advanced but none the less interesting. It is hypothesised that the two JAZ5 EAR domains contribute to JAZ5’s function as a dominant transcriptional repressor. The mutation created and transgenic lines generated will, when selected for homozygosity, help determine the role of these JAZ5 EAR domains, individually and in combination, in plant immunity.

In summary, the tools that have been produced for HUB37 and JAZ5 expression and Ear domain analysis, particularly the transgenic lines (and the accompanying glycerol stocks) provide a great foundation to characterise the role of these diverse EAR domain containing proteins in plant immunity. Moreover, having made the JAZ derivatives using GoldenGate, the option exists to go back and re-generate other constructs as necessary. For example, different epitope tags could be used and other genes could be inserted into final Level 2 construct. Level 0 JAZ5 gene mutations can be used as templates and amplified into different systems such as yeast two-hybrid for in vitro protein-
protein interaction or yeast 1 hybrid as is currently underway at Warwick University.

Further studies using more quantitative approaches and replicated experiments are needed to determine whether PTI/ETS impacts HUB37 abundance. The most obvious approach is to first test a variety of protease inhibitors and inhibitors of the 26S proteasome to see if these can prevent degradation of HUB37 during PTI, as well as undertaking in vitro supernatant mixing studies as described in detail above.

Overall, I feel it is fair to say that this study has made a significant contribution to both identifying a potentially novel role for HUB37 in plant immunity and generating a valuable set of tools to define the role of HUB37 and the HUB37 and JAZ5 EAR domains in contrasting mechanisms that are central to plant immunity.

Concluding Remarks

Due to personal circumstances, I decided not to relocate with my research group and therefore could not complete a PhD. However, I have generated an excellent range of tools and validated an interesting phenotype for HUB37 - providing a platform for others to follow up the results I achieved.
Supplementary Data 1 - At5g47390 – HUB37

Discovery of Hub37

Time series microarray dataset

- Infiltrate mature leaf tissue with:
  - DC3000
  - DC3000hrpA-
  - MgCl₂ (Control)
- Time course: 0, 2, 3, 4, 6, 7, 8, 10, 11, 12, 14, 16, 17.5hrs (13 time points)
- 4 biological replicates
- 2 technical replicates
- 156 samples using 312 arrays

Network Inference Modelling

Differential gene expression between DC3000 and hrpA treatments

Venn diagram showing the result from BETR analysis

~ 30000 probe sets
Significance = 0.01

10474
1005
1750

DE
TFs

Suppression of plant defense
Pathogen nutrition

Aryee et al. (2009) BMC Bioinformatics 10(1) 409

Slides from Murray Grant
Full length Genomic Sequence

1  AAATAAAAAA   AAAAAATCGG   CCAGATAAAT   CGAATTATG   TAATAAATCC
51  GACCAGATATA   ATGAGTATT   TTTGCTTTCT   TCGCTGCTCT   TGCTCCTCTA
101  TCTCTTTTCTC   ACAATTTAGAT   TCTGTCGTT   TTTCGAGTAC   AACTAAGATC
151  CGATCCGGCA   GCGTTTCGCA   CTTCGATCAC   ATCCGATTAAT   GAGAACAA
201  TCGGCGGCTCG   ATCGAATCCT   CGATCTTCTT   ACTGCAATCA   CAGCTGAGAT
251  AACTCTCGGA   CTGCCTCCAA   TCGGCGCTGG   AAGCTCTTTG   GTGTTTCGCT
301  CACCGAAGAGT   TCTCGATGGGT   AAATGCAGAA   TATGATCTCT   GAGAAAAAG
351  GACCAGATAA   ACTGATATTA   TTGTCTTTCT   TCCGCTCCTT   TGTCTCTCTA
401  GATTTTCTTTG   CCGTCTCTTT   CCTCTATCCG   CGAGAGAAG   AAAGTTATCT
451  TCTCTTTCTC   ACAATTAGAT   TCTGTGCTTC   TTCTGCGATC   AACTAAGATC
501  CGATCCGCGA   GC   GTTTCAGA   CTTCGATCAG   ATCCGATTAA   GAGAAGCAAA
551  TCTGGGTCGGG   TATGACTCGT   CGATGTTCTC   ACTGCAATCA   CAATGGCCAC
601  AACTCTCGGA   CTTTGTCCCAA   TCGGCGGCTGG   AAGCTCTTTG   GTGTTTCGCT
651  CACCGAAGAGT   TCTCGATGGGT   AAATGCAGAA   TATGATCTCT   GAGAAAAAG
701  GACCAGATAA   ACTGATATTA   TTGTCTTTCT   TCCGCTCCTT   TGTCTCTCTA
751  GATTTTCTTTG   CCGTCTCTTT   CCTCTATCCG   CGAGAGAAG   AAAGTTATCT
801  TCTGGGTCGGG   TATGACTCGT   CGATGTTCTC   ACTGCAATCA   CAATGGCCAC
851  AACTCTCGGA   CTTTGTCCCAA   TCGGCGGCTGG   AAGCTCTTTG   GTGTTTCGCT
901  CACCGAAGAGT   TCTCGATGGGT   AAATGCAGAA   TATGATCTCT   GAGAAAAAG
951  GACCAGATAA   ACTGATATTA   TTGTCTTTCT   TCCGCTCCTT   TGTCTCTCTA
1001 TGTAAGATCTG   TTGGGG   TTCT   AATTGATATG   CTTGAGGATA   TCATTTTGTA
1051 GCCAATCCTG   CTTAAGCATC   TTGCTTCTCT   CATGCTGAGA   TAGGTGGATT
1101 ATGTAATCTC   CTTCTTATTAT   TCTGCTTATG   CTGTGCTTCTG   TGCTCCTCTA
1151 GGTGGAGATAT   ATCCCAAGGG   ATTTGAGAAG   ACCAGAGGAA   GATAATATTC
1201 CTGGTGAAAC   TGAAATGCAA   GTGCTGACT   CTATTCATCA   GACACTTGCT
1251 CTAGCTCAC   TTCACGCACC   GTCAATCTTG   GAAATCGAAG   AATGTGAATC
1301 CGATCCGCGA   GCGTTTCGCA   AAATGCAGAA   TATGATCTCT   GAGAAAAAG
1351 TCTGGGTCGGG   TATGACTCGT   CGATGTTCTC   ACTGCAATCA   CAATGGCCAC
1401 AACTCTCGGA   CTTTGTCCCAA   TCGGCGGCTGG   AAGCTCTTTG   GTGTTTCGCT
1451 CTACTTTTCA   CCATATTACC   CGTTTCCATT   CCAATATGG   CCGCTGCTCT
1501 ATGTTCTCTGA   ACCACCAAAG   AAAGAGGAAA   CTCAATGAAT   TCTCAGACCA
1551 ACTGTGGTGCT   ATCGAAAGGC   TCTCATCAAT   GTGAGCTGAC   TGCTCTCTAT
1601 GTCTAAGCTC   AGCTTTGAGG   AGTCACAAGG   ACATGGAGAA   TCCAGAAGG
1651 CTCATTCTAT   GAAGCTGAGA   GGCGGTCTAT   CTGCTGAGAG   ATGACACTTT
1701 CACCCGAATTC   CTAGCTCTGGA   TAGTTCGACAG   ATCAAAGGCG   TGATACACGC
1751 TTTATAAAGAG   ACCTGAGAAGG   GTGATGTGCT   AAAATGGGAA   CTTGATTTGG
1801 GTTACACGGTT   TAGTGTGTGGG   TCCAGTAAAC   TTAATAAGTC   TTCTCCTTGT
1851 TAGGTGGGGT   ACTTGGGTTA   GGATGTTTATG   GTTCAGCTTT   GATCATATTG
1901 GAAAGAAAAA   AGAAGAAAAA   AAGGGAGAAA   AAACAAAATA   TTATTTTCTG
1951 CCTTACATTTC   CTATTTATTG   TAGGCTTTCT   TTTCGACTCT   AGGATGCGCT
2001 ATTTTCGGTT   TAATCTGTAAG   TAAAATTTAG   AATTATTTAG   TTTGGAATAA
2051 ATAAAAATCAC   AGTTTGTCTT
Gene Sequence – EAR domain highlighted

ATGACTGTCG GATGTCTTCTCA CTGCAATCAC AATGGCCACA ACTCTCGGAC
TGTCCTCAAT CGGCCGCTGA AAGTCTTCTGA TGTTCCGGTC ACCGAAGGTG
CGATCCGGAA AAGTGCAAGT ATGGGTAATTC TAGCCATTA CACGGGTTCT
GGATCGGGGT GGCATGGAAC CGGGTCCAAC ACTCCGGGTG TCCTCGGGTA
TGTCCTCAGG CATGTCGCTG TPGATGGGTA CGTTTCTGAG GATTTCTGTTG
CTGCTCTTCC CTTAGCCCAG GAGAGAAGAG AGGATGTCTT TATTAGGTTTT ACAGAAGCTG
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TCACGCACC TCAATCTTGG AAATCGAAGA ATGTGAATCA ATGGACTCCA
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GCCGCAACTT CTCGGCTCAT CCCCCCATATC ATATCCGACC TACCTTTCAC
CATATTCCG GTTTCATCC CCAATATGGC TGCTTGTTA TGTCTGTAAG
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AAGCTA GGGTGTCATC TTTCAAGACAA TCGCATTTCC ACCGGAAATCC
TAGCTCTGAT AGTTCACACA TCAAAAGCCGT GATACACGCT TTA TAA

ISI predicted binding sites
At5g47390 contains conserved regions:

- a CCHC-type zinc finger ‘RCSHCNH NGHNSRTCPN RG’ with an R/KLFGV-type repression domain ‘KLFGV’;
- a R1MYB domain ‘PWTE EEHRMFLGL QKLGKGDWRG ISRNYVTTTR PTQVASHAQ YFIR’ with a nuclear localization signal ‘ERKK’ and ‘SRRKRRS’;
- a putative leucine-rich nuclear export signal (NES) ‘LSLKL’;
- an EAR-like domain LxLxL (Hiratsu et al., 2003) that has the potential to act as a transcriptional repressor domain ‘LSLKL’;
- and, five low complexity regions.
Schematic diagram of HUB37 Protein
Homology structure of HUB37 biased on TRF2 a human MYB transcription factor (34%). Image from Nature (date accessed 19 January 2016)
http://www.nature.com/ncomms/2014/140507/ncomms4767/extref/ncomms4767-s1.pdf

Supplementary Data 2 - At1g17380

Supplementary Data 2, At1g17380 – JAZ5

Full length Genomic Sequence
(Complementary strand)

1  ATGAGCTATT GAGCTAGTAG CCTCTTGATAC TCTTCCATTTC TACGCGCAAT
51  CCACGCACCA ACAAAAAGAA AAGAAAAGAA GAGATAAAGA ATATCTTTAA
101  AAAGTAAGTG TGGAGAATTC TTTCTTCTCA ATAAACAAAC ATATGTCGTC
151  GAGCAATGAA AATGCTAAGG CACAAGCAGC GGAGAAATCT GACTTTACCC
201  GGAGATGTAG TTTGCCTCAGC CGTTACTTGA AAGAGAAGGG TAGTTTCGGA
251  AACATTGATC TTGGCTTATA CCGAAACCC GATTCCAGTC TCGGTTGCC
301 CGGAAAATTC GATCCACCAG GTACTTTTAT TATCTTTTTC TTCTTTCCATG
351 GCCGCCACTT GGTAAATGTA CTTTTAAAAC TCAGTCTCGT TACACTTTGC
401 TTTTGCCAGA TATCTACTTT CTTGTATTGG TCTTTCAATT TAAGTCAATTT
451 CCTTACTTCA GAATAATTTT CTCCTTGTAAT TTATGAGAAC AAGTAACAAA
501 AATAACGAAT AGAGGATAT AGAAATTGAT TGAGATGTTG TAAATAAAAT
551 TCCAAAGATA TAAGATGCTA TATACATTTT CTAAGATTTT TATAGTTCAAC
601 GAAAGTTTTTT ATTCTCTTTTA TGCAAGGAAA CAAATGGCGA TGCATAAGGC
651 AGGGCATTCCC AAAGGGCAAC CCTCTACCTC ATCAGGAGGC AAAGTCAAGAG
701 ATGTTGCTGA CCTCAAGTCT CTCTTTTTGT TCTTTGGGAA TACCTTGTGT
751 TTTTGTTTAT TGGAAGATAG ATGGAGTTAA TCCGGTTAAA
801 ATTTGTTTTT GGTCTGAATC TGGTTTTTGT TTGCAGTGAA TCAAGCCAG
851 GAAGTTTCCGA GCTGACCATA TCTTTGGGAG GAAAGTTTTT AGTATATAAT
901 GAGTTTCCCG TAGAAAAGCC TAAAGAGATT ATGGAGTAG CAAACAAGGC
951 CAAGCCCTGTG ACTGAGATTAA ACAATCGAAT ACCAACAATA GACGAAAACA
1001 ACAACAACAA GACGAGCATG GTTCTTTCTG ATCTCAATGA GCCCTACTGAT
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1101 GTGGGAACGT ATAGCACTGA GAGCTCCCTC CCACTGATTT TTTGCTAAAC
1151 GAAAGACAG GTATTTAACCC TTATCATACT TTTGGAACCT TGTTTTTAATG
1201 TTCCAAATTT CAAATCCTCT TAGTTAGTTT AAAACTCAA AAGTTAAAGGA
1251 TCTTCCCTCT GATAAAGGTT CTCTAAAACT ATCCTCAACA AGCCTCCTAA
1301 TTTACGTTTAT TAATTTTTCT GTGGCAAGGC TGTTGAGGG GCCTCGTACC
1351 GAAGTTAACAA AAACCCAGGT CATCTCGTTT ATCCTGCCAA GCCAGAGATT
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1651 CTACAGTATG TAATCTTTAT CTAACCTGAA TATTCATTCA TAAACACAAAT
1701 AGACGATAGT AAAGTATAT ATATAA
At1g17380.1 contains four conserved domains: (information from http://www.biomedcentral.com/content/pdf/1752-0509-4-43.pdf (date accessed 19 January 2019))

- **TIFY** domain - This short possible domain is found in a variety of plant transcription factors that contain GATA domains as well as other motifs. Although previously known as the Zim domain this is now called the tify domain after its most conserved amino acids. TIFY proteins can be further classified into two groups depending on the presence (group I) or absence (group II) of a C2C2-
GATA domain. Functional annotation of these proteins is still poor, but several screens revealed a link between TIFY proteins of group II and jasmonic acid-related stress response.

- **Divergent CCT motif** - This short CCT_2 motif is found in a number of plant proteins. It appears to be related to the N-terminal half of the CCT motif. The CCT motif is about 45 amino acids long and contains a putative nuclear localisation signal within the second half of the CCT motif.

- **EAR domains** – transcriptional repressor domain found in plants

- **Low complexity region** - the genetic mechanisms from which they arise lends them remarkable degrees of compositional plasticity (Coletta, et al., 2010)"

---

**Amino Acid composition**

![Amino Acid Composition Chart](image-url)
Supplementary Data 3 - Method

Cloning strategy for C1-35Spro:MYB:3XHA-NOS, hemagglutinin sequence

Triple hemagglutinin (HA) sequence from pH1-HA3: ATG – start codon; GREEN – Protein sequence
DNA sequence:

```
CC ATG GCA GGT TAC CCA TAC GAC GTT CCT GAC TAT GCG TCA CTC TAC CCC TAT GAC GTA CCG GAT TAT GCA TCC CTA TAT CCG TAT GAT GTT CCA GAT TA C GCT TCT AGA GGC GTC CAC CAT ATG
```

Protein sequence:

```
MetAGYPYDVPDYASSLYPDYPDYSLLYPYDVPDYASLRSSRGVHHeMet
```

Golden Gate Cloning

JAZ5 – EAR Domain Mutagenesis.

Highlighted primer target change (mismatch) and EAR (Ethylene-Responsive Element Binding Factor-Associated Amphiphilic Repression Motif-Containing Transcriptional Regulators).

```
AT1G17380.1
gattttttttttttggtttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Golden Gate plasmids used in Cloning

Rotate Figure on its own page

A Golden Gate Modular Cloning Toolbox for Plants

ACS
2014

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**Level 0**

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Terminator or vector | pICH44300 | spectinomycin | A12 | 3'UTR, polyadenylation signal/terminator, act2 (A. thaliana) 5
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**Level 1**

| Level 1 | P1 acceptor | pICH47732 | carbinicillin | B3 | level 1 receptor, position 1, forward orientation
|---|---|---|---|---|---
| Level 1 | P2 acceptor | | | | level 1 receptor, position 2, forward orientation

| Level 2 | acceptor | GBKT7 | |

**Primer List**

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85
Supplementary Data 4 - Results

Characterise the function of HUB37 in plant defence and selecting homozygotic lines.

To select homozygous complementary and overexpressing HUB37 lines with different epitope tags to identify protein interactions:

– Col_0 and hub37 pC1::35Spro:MYB:3xHA

– Col_0 and hub37 pC1::35Spro:MYB:GSTag

MYB Transgenic Seed Selected on hyg plates

Growth curve and western blot analysis in main text, Figure 3.3.
HUB37 Expression Decreases Over Time When Plants are inoculated with DC3000

Repeat experiments for HUB37 protein degrading in planta over time when inoculated with DC300.

**in vitro HUB37 degradation rate**
Figure 5.5.1
Degradation of HUB37 in vitro over time; Col-0 DC3000 inoculated protein extract was mixed in a one to one ratio with non-inoculated HA:HUB37 expressing lines. Expression of HA:HUB37 too low to visualise in the Col-0 overexpressing background. Clear degradation can be seen in the hub37 background. Challenged protein mix appeared to degrade more by 18 h than compared to non-inoculated tissue.

**Determine if the EAR motifs in JAZ5 act as the functional repressor.**
JAZ5 contains both LxLxL and DLNxxP in the C-terminal and middle region respectively. The repressor activity of the EAR motif found in JAZ proteins is currently unknown

Complementation analysis of JAZ5 and JAZ10 in jaz5/10 background.

**Method**

**Expression - Plants selected with BASTA (1:500).**

Previous work showed that At:jaz5/10 plants exhibited more chlorosis in comparison to At:jaz10 plants suggesting that JAZ5 and JAZ10 act synergistically.

**Function - Two leaves from JAZ5pro:3xMYC:JAZ5 and JAZ10pro:3xMYC:JAZ10 were inoculated with DC3000: 0.002 for 5 days**
- Expect to see:
  - Col_0 
  - jaz5/10 bit chlorotic
  - jaz5/10 pB8::JAZ5pro:MYC:JAZ5 very chlorotic
  - Jaz5/10 pB8::JAZ10pro:MYC:JAZ10 Similar to Col_0
  - Even more similar to Col_0
Results: Phenotype

Two leaves per plant were inoculated with PtsD where possible. Some plants died – plants without BASTA resistance which are the gaps. Some every small so only one leaf possible to inoculate.
References


Data derived from the PRESTA consortium (reference Lewis et al., 2015)


Siddharth Jayaraman, Marta de Torres per com


