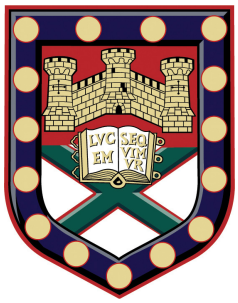

The role of Jasmonate-ZIM-Domain proteins in Systemic Immunity

Submitted by Daniel Wise to the University of Exeter
As a thesis for the degree of
Masters by Research in Biosciences
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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……Dwise……..

Abstract

Phytohormone signalling has been shown to have crucial and broad roles in plant development and survival. One of these many roles is plant immunity and response to pathogen invasion. Three main hormones salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA), have been shown to have a dramatic impact on plant immunity and determining susceptibility or resistance to pathogen attack. Post invasion defence responses are primarily regulated by a SA dependent pathway. SA mediated defence is shown to be compromised by antagonistic action of the JA and ABA signalling pathways. The multifaceted crosstalk between these three hormone signalling pathways dictates plant susceptibility and resistance. In this study we focus on the regulation of the JA signalling pathway, in particular the role of the transcription factor inhibitor, jasmonate-ZIM-domain proteins. An active form of JA, jasmonoyl–isoleucine (JA–Ile) facilitates the interaction between COI1, an F-box protein and various JAZ proteins to form SCF E3 ubiquitin ligase complexes. SCF^{COI1} then tags the captive JAZ proteins for ubiquitination by the 26S proteasome, this allows for transcription of early JA-related genes. We challenged *A. thaliana* strains, with specific JAZ protein insertional mutations, with the model pathogen *P. syringae DC3000*. From this work we show a role for JAZ proteins in host susceptibility and resistance. In particular we show a decreased ability in *P. syringae* to proliferate in *A. thaliana jaz* 7 knockout mutants, as well increased susceptibility to *jaz* 5/10 deficient mutants. We also identify a positive relationship between coronatine associated gene expression and *jaz* 5/10 deficient mutants.

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1: Introduction

The study of plant microbial interactions and the mechanics of pathogen virulence are large and vital areas of research, due to high economic importance and the pivotal role this research has in maintaining food security.

Plants are under frequent attack from many different organisms. Plants of particularly high economic value are no different and can be devastated by a particularly virulent strain of pathogen.

Pseudomonas syringae, Phytophthora infestans, *Magnaporthe oryzae* to name a few infect valuable cultivated crops, tomato, potato and rice respectively and can have drastic effects on crop yields (Jones et al. 2006). In extreme cases pathogens can effect the food supply to a whole country, as was seen in the Irish potato famine in 1845.

Although pathogen attack can have dramatic effects on food sustainability plants are not helpless in responding to pathogen stresses.

1.1: Plant innate immunity. Pathogen associated molecular pattern triggered immunity and effector triggered immunity.

To combat pathogen attack the innate plant immunity arsenal consists of two characterised methods, the recognition of Pathogen/ Microbial Associated Molecular Patterns and the gene for gene relationship.

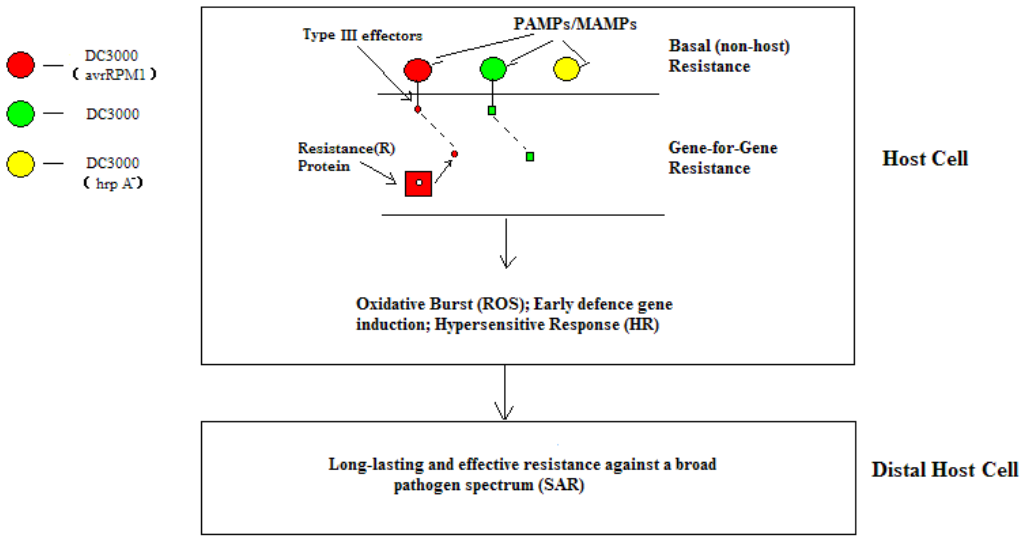


Figure 1. Immune response induction model, the model details the host's two defence mechanisms activated during specific pathogen attack. This figure illustrates the appropriate immune response when invaded by three variants of the plant pathogen *P. syringae* strain DC3000. DC3000 expressing avirulence factor Avr*RPM1*, a *HrpA* deficient mutant and DC3000 absent of Avr*RPM1*.

The first method is the detection of Pathogen/ Microbial Associated Molecular Patterns (PAMP's or MAMP's). These are small molecular motifs that are recognised by Pattern Recognition Receptors (PRR's) such as Toll-like receptors (TLRs) (Jones et al. 2006). Well-known examples of PAMP's are located to flagellin, peptidoglycans and fungal chitins. The detection of these PAMP's induces PAMP Triggered Immunity (PTI) (Muthamilarasan & Prasad. 2013).

Effective PAMP's are complementary to conserved coding regions integral to the pathogens overall fitness or effectiveness to be virulent. The advantage of recognising these highly conserved regions is that the pathogen is unlikely to retain its fitness if these regions are altered during evolution.

Effective PAMP's are as discussed previously, flagella (Wei et al. 2012; Verma et al. 2005). This is a whip like structure integral for movement protruding from the cell body in flagellate bacterium. This a particularly good PAMP as any interference to the flagellum could lead to a loss of motility and therefore a

decrease in fitness, whereas if the bacterium fails to act, the bacteria will be detected and subsequently destroyed by the initiation of PTI. Chitin is another example of an effective PAMP which is found in the cell walls of fungi and is the main source of structural integrity in many fungi, making chitin another well conserved site of detection (Newman et al. 2013).

While PTI is an effective method of recognising pathogen invasion many pathogens evolve approaches to avoid detection, as well as methods to disrupt the PTI response (Fujikawa et al. 2012; Sánchez-Vallet et al. 2013). Natural selection favours the survival of these more successful pathogens, illustrating the necessity of having other methods of initiating an immune response.

The second method of defence is the detection of secreted virulence factors first proposed as the gene for gene hypothesis (Flor. 1971), later known as the gene for gene relationship. This showed that the inheritance of both resistance in the host and the pathogens ability to cause disease is controlled by pairs of matching genes (Falk et al. 1999). The plant carries a gene referred to as a resistance (*R*) gene. The paired pathogen gene is called an avirulence or *Avr* gene. Plants with a specific cognate *R* gene confer resistance to a pathogen carrying a corresponding effector gene, the virulence factor that allows the pathogen to be recognised and prompts an immune response is then known as an avirulence (Avr) factor. This immune response is referred to as effector triggered immunity (ETI) (Jones et al. 2006).

The gene for gene hypothesis works on the assumption that there is a direct interaction between the proteins encoded for by the *Avr* gene and a specific corresponding *R* gene. Flor's discovery has been continually built upon. In 1977 Keen and Bruegger proposed the elicitor receptor model an adaptation of the

surface carbohydrate elicitor receptor model from Albersheim and Anderson-Prouty in 1975. The elicitor receptor model suggests the pathogens *Avr* protein either directly or indirectly produces a signal, known as the elicitor, which is then detected by a sensor or receptor protein coded for by the hosts sensor gene (Prell & Day. 2000).

Understanding progressed further as *Avr* and *R* genes were characterised. The first Avr being identified in 1984 by Staskawicz and co-workers, the Avr dubbed Avr*A* was isolated from *Pseudomonas syringae* pv*. Glycinea* (Staslawicz et al. 1984).

In 1990 an article was contributed to Nature linking the highly conserved avirulence gene Avr*Bs2* found in *Xanthomonas campestris* pv*. Vesicatoria,* to a negative effect on fitness when challenging the host pepper (*Capsicum annuum*) with the *Bs2* gene (Kearney & Staskawicz. 1990). This finding provided a potential explanation as to why hosts expressing the *Bs2* gene confer the only effective field resistance found to *Xanthomonas campestris.*

The theory of interacting *Avr* genes and resistance genes was further expanded on. Surmising that the R proteins interact with or guard a protein known as the guardee, which is the target of the avirulence protein (van der Biezen & Jones. 1998). When the guard protein detects modification to its guardee protein, such as cleavage to the guardee protein, an immune response named the hypersensitive response (HR) is induced (Dangl & McDowell. 2006).

The guard hypothesis has been supported experimentally; the *Rpm1* gene in *Arabidopsis thaliana* was shown to respond to two completely unrelated avirulence factors (Avr*Rpm1* & Avr*B*) during *P. syringae* infection (Mackey et al.

2002). The guardee protein RIN4, is targeted by the two aforementioned avirulence factors Avr*Rpm1* and Avr*B* for phosphorylation, presumably the targeting of RIN4 for activation is linked to the role of RIN4 as a negative regulatory factor of PTI (Afzal et al. 2011). The phosphorylation of RIN4 activates RPM1 by means of Nod-like receptors (NLRs) (Chung et al. 2011; Liu et al. 2011; Innes. 2011) resulting in the induction of the HR.

Yeast two-hybrid studies of the tomato Pto/Prf/AvrPto interactions have shown that the Avr protein, AvrPto, interacts directly with Pto despite Pto not having a leucine rich repeat domain (LRR). This makes Pto the guardee protein (Zong et al. 2008), which is protected by the nucleotide binding site-leucine rich repeat (NBS-LRR) protein Prf. However, *Pto* is a resistance gene alone, which provides argument against the guard hypothesis, suggesting greater complexity in gene for gene resistance (Gabriëls et al. 2007).

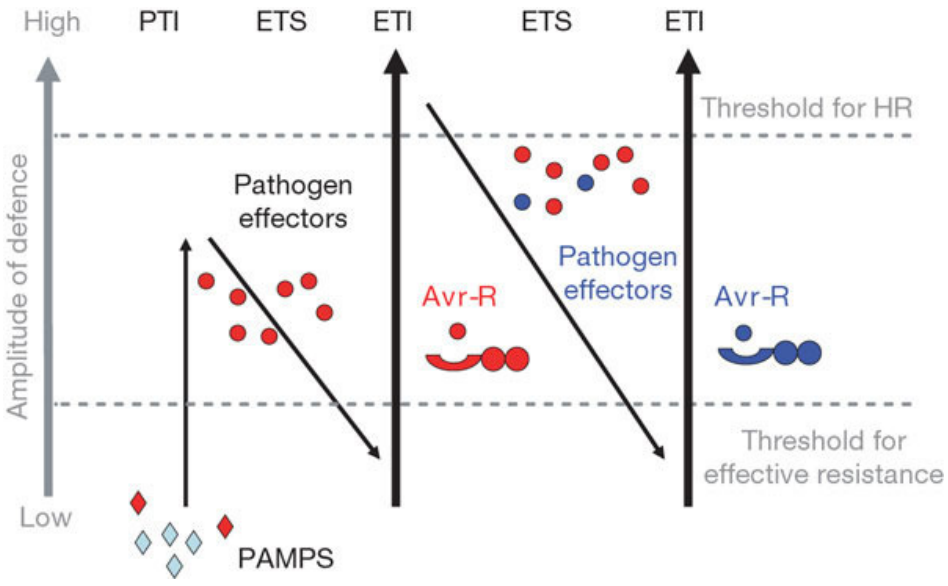


Figure 2. Zig-Zag model of plant innate immunity. This diagram displays the pathways of pathogen recognition conferring effective resistance via PAMP triggered immunity (PTI) and effector triggered immunity (ETI). The suppressive effect of pathogen effectors proteins on host defence mechanisms is also shown, effector triggered susceptibility (ETS) (Foodsecurist 2014).

1.2: Hypersensitive response

The hypersensitive response (HR) is a form of programmed cell death triggered following the recognition of pathogen-associated molecules. The HR is characterised by the rapid death of cells in the local region of infection. This response acts to limit infection by biotrophic pathogens, containing the infection to the site of detection therefore restricting the infection from spreading (Heath. 2000).

The Hypersensitive response generated by gene for gene interactions takes place in two stages. Firstly the activation of the *R* gene triggers an ion flux, involving an efflux of potassium and hydroxides outside of the cells and an uptake of calcium and hydrogen ions into the cell (Heath. 2000). However, in certain cases it has been shown that PTI can initiate a HR (Gimenez-Ibanez & Rathjen. 2010)

The second stage consists of the cells generating an oxidative burst by generating reactive oxygen species (ROS), superoxide anions, hydrogen peroxide, hydroxyl radicals and nitrous oxides (Morel & Dangl. 1997). These compounds affect the cellular membrane function in part by inducing lipid peroxidation and by causing lipid damage (Heath. 2000). The alteration of ion components in the cell and the breakdown of cellular components in the presence of ROS result in the death of affected cells, causing the formation of local lesions. This can be observed on an *A. thaliana* leaf six hours after infiltration with *P. syringae* DC3000 Avr*Rpm1* (Mackey et al. 2002).

1.3: *R* gene classification

There are several different types of *R* gene, the majority of *R* genes are nucleotide binding site leucine rich repeat (NBS-LRR) genes (Boller & Felix. 2009). The protein products of these *R* genes contain a nucleotide-binding site (NBS) and a leucine rich repeat region (LRR) (Boller & Felix. 2009). From here *R* genes are divided again into two more classes. One class has an amino-terminal Toll/Interleukin 1 receptor homology region (TIR) (Bernoux et al. 2011). This class includes the N resistance gene of tobacco (*N. tabacum*) against tobacco mosaic virus (TMV). The other subclass does not contain a TIR region but instead has a leucine zipper region at the N-terminus. The protein products encoded by these classes of resistance gene are found within the plant cell cytoplasm (McDowell & Woffenden. 2003).

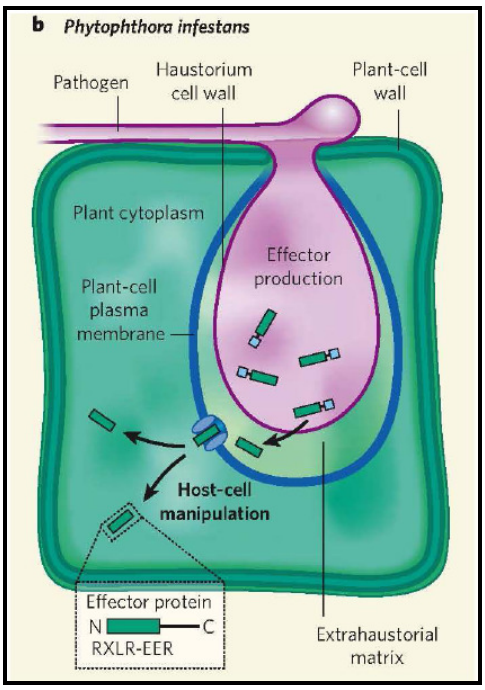


Figure 3. A classic model of plant microbe interactions, showing how effector proteins are delivered into the host cell. This example is of *P. infestans* infiltrating a potato plant (*Solanum tuberosum* L) (Talbot NJ. 2007).

In addition, there is also an extracellular LRR class of *R* genes. This includes the rice *Xa21* gene for the resistance against *Xanthomonas oryzae* and, the *cf* genes of tomato that confer resistance against *Cladosporium fulvum* (Chen et al. 2014; Liebrand et al. 2012). These proteins have a classic receptor-kinase format, which is an extracelluar LRR, a membrane spanning region and an intracellular protein kinase domain (Ellis et al. 2000). The unique extracellular domains of this class of resistance genes play a particularly important role enabling the detection of extracellular pathogens (Ellis et al. 2000; McDowell & Woffenden. 2003).

1.4: *P. syringae* infection and the role of effector proteins.

The model organism, *Pseudomonas syringae* pv. tomato is an epiphytic rod shaped, Gram-negative bacterium with polar flagella. DC3000 is the wild type strain, which is virulent to a wide range of plant species including *A. thaliana* (Xin & He. 2013).

P. syringae infiltrates its host and then during the infection process uses effector proteins to suppresses basal defences and redirect the host's metabolism to support *P. syringae* growth and development (Rico & Preston 2008; Collmer et al. 2000; Büttner & He. 2009). To do this the bacterium secretes effector proteins or virulence factors using a specialised infection structure known as the type III secretion system (T3SS) (Galán & Wolf-Watz 2006). The T3SS is comprised of structural and non-structural proteins; the structural proteins assemble the needle complex (NC) (Chatterjee et al. 2013). The NC comprises of a membrane-embedded basal structure, an external needle known as the

pilus that protrudes from the bacterial surface through the plant cell wall and a tip complex that caps the needle. Upon contact with the host cell a translocon is assembled between the needle tip complex and the host cell, providing a gateway for the delivery of type III effector proteins (T3Es) (Chatterjee et al. 2013).

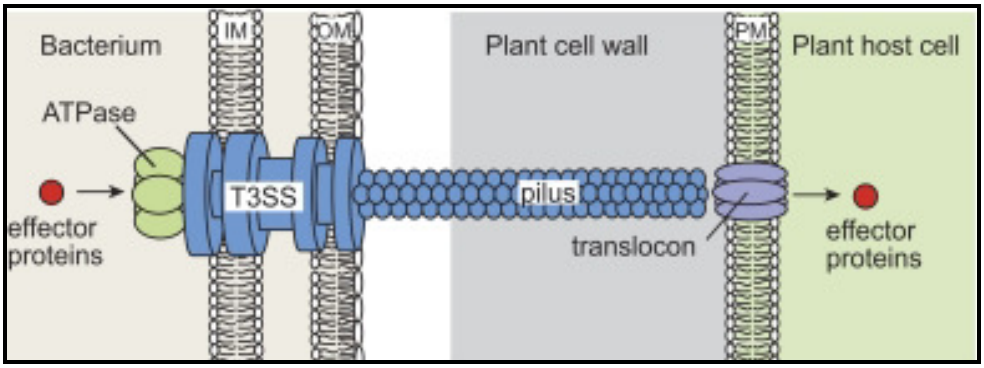


Figure 4.The secretion apparatus spans the bacterial membranes and is associated with a cytoplasmic ATPase. The T3SS from plant pathogenic bacteria is connected to an extracellular pilus that spans the plant cell wall, which serves as a transport channel for secreted proteins. The pilus is linked via the tip complex to the translocon, which forms a channel into the host plasma membrane, allowing transportation of effector proteins into the host cell cytosol (Büttner D & He SY. 2009).

In addition to delivered effector proteins, toxins are also secreted into the host to aid infection. A documented example of this is coronatine (COR) (Mitchell. 1982). COR is a low molecular weight phytotoxin and Jasmonic acid mimic (Geng et al. 2012**)**. COR is comprised of two separately created sub-units coronafacic acid (CFA) and coronamic acid (CMA), which are then fused together by an amide bond between the amino group of CMA and the acid group of CFA (Bender et al. 1999; Brooks et al. 2005; Völksh et al. 2005).

Coronatine has been associated with numerous roles in the infection process. One such role is overcoming stomatal defence (Zeng et al. 2010). Stomatal openings have long been shown to be a major route for bacterial entry (Huang

1986), with more recent studies presenting an active role for stomata limiting bacterial infection and stomatal closure being linked to PTI (Melotto et al. 2006). Coronatine is a notable example of virulence factors that interfere with stomatal closure (Zeng et al. 2010; Zeng et al 2011). Coronatine has also been shown to be important in promoting bacterial proliferation in the apoplast (Uppalapati et al. 2007), inducing disease susceptibility in systemic tissue (Cui et al. 2005) and the activation of jasmonate (JA) signalling (Melotto et al. 2008; Thines et al. 2007).

1.5: Systemic acquired resistance and systemic induced susceptibility.

As previously discussed *P. syringae* DC3000 Avr*Rpm1* induces an immune response in *A. thaliana* but when a HR has taken place a secondary resistance response known as systemic acquired resistance (SAR) is induced in distal, uninfected tissues (Mou et al. 2003).

SAR provides a long-lasting resistance throughout the plant to any subsequent infections by a broad range of pathogens. SAR is also referred to as the broad-spectrum response. SAR is associated with the induction of a wide range of genes known as pathogenesis-related (PR) genes, which show elevated expression in distal leaves (Mou et al. 2003).

The activation of SAR requires the accumulation of endogenous salicylic acid (SA); SA induces the reduction of oligomeric disulfide-bound NPR1 (non-expressor of pathogenesis-related genes), which is a central regulator of SAR. Active NPR1 monomers migrate to the nucleus and interact with members of

the TGA transcription factor family as part of the transcriptional reprogramming associated with SAR (Despres et al. 2003).

Recent studies have shown that the translocated signal that induces SAR might be a lipid derived molecule potentially jasmonates or MeSA (methyl-salicylic acid) (Park et al. 2007). In contrast to SAR, a study published in 2005 reported that high doses of virulent strains of the bacteria *P. syringae* will induce susceptibility to subsequent infection. This occurrence has been called systemic induced susceptibility (SIS) and is in direct opposition to the well-studied phenomenon SAR (Cui et al. 2005).

1.6: Hormone signalling in innate immunity

Plant signalling has been shown to play many roles in a variety of biological functions, including plant development and responding to an assortment of biotic and abiotic stresses (Imber & Tal. 1970; Verhagen et al. 2004; Wang et al. 2010). The initiation of these pathways is mediated by phytohormones such as abscisic acid (ABA), salicylic acid, ethylene (ET) and jasmonic acid (JA) (Falk et al. 1999, Torres-Zabala et al. 2007; Wasternack & Hause 2013).

1.6.1: Abscisic acid

Abscisin II now known as ABA is a terpenoid hormone. ABA was first identified in cotton whilst investigating abscission (Addicot. 1963). ABA is formed from its

precursor zeaxanthin, which is synthesised into xanthoxin. Xanthoxin is subsequently transported to the cytosol where it is oxidised, forming ABA (de Torres-Zabala et al. 2007).

ABA has been shown to have a multitude of roles in plant physiology including promotion of seedling growth (Thomas et al. 1965), water retention (Imber & Tal. 1970; Tal et al. 1970) and stomatal closure (Jones & Mansfield. 1970). Although more recently ABA has been shown to play a role in abiotic stress responses (Cutler et al. 2010). ABA has a diverse role in pathogen attack and the affects of ABA on pathogenicity have been shown to be pathogen dependent. For example ABA confers resistance to fungi and oomycetes but contrarily promotes susceptibility during herbivore attack and *P. syringae* infection (de Torres-Zabala et al. 2007).

1.6.2: Salicylic acid.

Salicycic acid is a phenolic phytohormone. SA is synthesised from phenylalanine but is also produced in a phenylalanine independent pathway in *A. thaliana* (Loake & Grant. 2007). SA has roles in plant growth and development including photosynthesis and transpiration (Manthe et al. 1992; Wang et al. 2010; Manthe et al. 1992). SA also has strong links to innate immunity and disease resistance, it is known that subsequent to pathogen attack and during HR associated cell death SA is accumulated (Falk et al. 1999; Jirage et al.1994). Active forms of SA are formed either by conjugation of amino acids or methylation (MeSA), these compounds have been shown to play roles in plant defence response regulation (Fu et al. 2012; Loake & Grant. 2007).

1.6.3: Jasmonic acid.

Jasmonic acid (JA) is the prototypical member of a class of related oxylipin signalling molecules (collectively referred to as jasmonates) (Schaller et al. 2005). Jasmonates are cyclopentanone derivatives and are derived biosynthetically from fatty acids. Jasmonates are biosynthesised from linolenic acid by the octadecanoid pathway (Schaller et al. 2005; Turner et al. 2002). The JA pathway is involved in regulating a diversity of roles including stress responses and developmental processes; such as pollen viability, fruit ripening, root growth, cell cycle progression, tendril coiling and stress responses from pathogen attack (Wasternack & Hause 2013; Avanci et al. 2010; Browse. 2009; Creelman et al. 1992; Farmer. 2007).

The function of JA in defense was first proposed by Farmer and Ryan (1992), who provided evidence for a causal link between wounding caused by insect herbivores and the formation of jasmonic acid. Two main active forms of JA that have been characterized are firstly, methylated JA (MeJA), which has been shown to have varied roles in plant growth and development. Including root growth, flowering, seed germination and senescence (Wasternack. 2007; Yue et al. 2012; Berger et al. 1996). It is documented that MeJA accumulates in wounded plant tissue subsequent to pathogen attack as well as modulating wound gene expression (Creelman et al. 1992; Xu et al. 2003; Berger et al. 1996). The second active form is JA conjugated to the amino acid isoleucine (Ile) to form JA-Ile, which has been shown to play a pivotal role in JA signalling and Jasomonate-ZIM domain protein degradation (Thines et al. 2007) (see chapter 1.7).

1.6.4: Phytohormone signalling Cross talk

Plant hormone signalling is a complex subject. Not only are the mechanisms and roles of each individual signalling pathways broad in terms of function with seemingly overlapping roles but they are also incredibly variable depending on stimuli (de Torres-Zabala et al. 2007; Truman et al. 2007). For example in response to stress signalling varies to the type of stress for example biotic or abiotic. If the stress is pathogen based stress then the type of pathogen and the effector proteins at the disposal of the chosen pathogen strain affect the signalling induced. The sheer level of variability makes hormone signalling a difficult web to untangle and the signalling system has been shown to be even more complex due to the fact that each signalling pathway should not be considered as a stand-alone system (Derksen et al. 2013; Halim et al. 2006;). There have been numerous reports of cross talk between particular signalling pathways in response to a variety of stresses (Derksen et al. 2013; Kunkel et al. 2002).

For example we know that JA is a central signal of the broad-spectrum induced systemic resistance (ISR) (Verhagen et al. 2004), which is typically similar to classical SAR. Furthermore we know that JA is considered to antagonise SA-dependent responses and *vice-versa* (Spoel et al. 2003). Studies have shown that there is a rapid transient generation of JA signalling at the early phase of systemic responses to the HR, which is SA independent (Truman et al. 2007). Research suggests that SA and JA act synergistically in SAR though are separated temporally (Truman et al. 2007).

1.7: Jasmonic acid signalling and the role of Jasmonate-ZIM-domain proteins.

The interactions of *P.syringae* and *A. thaliana* are well studied, however in 2007 a new group of twelve plant specific proteins called Jasmonate ZIM-Domain Proteins (JAZ's) were discovered in *A. Thaliana* (Yan et al. 2007; Thines et al 2007**;** Chini et al. 2007). Adding a new dimension to the JA signalling pathway and more potential targets for pathogen effectors.

JAZ proteins belong to the TIFY family named after the conservation of the TIFYXG sequence found in the ZIM motif. JAZ proteins share two conserved motifs, ZIM and Jas (Staswick. 2008). The ZIM motif is thirty-seven amino acids long and found in all of the TIFY family. Where as the Jas motif is found at the C- terminus and includes a unique twenty-six amino acid long conserved region only found in members of the JAZ family (Yan et al. 2007; Thines et al 2007**;** Chini et al. 2007).

JAZs are a crucial link in the JA signalling pathway (Demianski et al. 2012; Pauwels & Goossens. 2011). In the first step of JA signalling, a small amount of the jasmonic acid precursor hormone that has accumulated, is attached enzymatically to molecules, such as L-isoleucine, producing an active ligand, jasmonoyl–isoleucine (JA–Ile) (Schaller et al. 2005; Thines et al. 2007). JA–Ile facilitates the interaction between COI1, an F-box protein and various JAZ proteins to form SCF E3 ubiquitin ligase complexes (Chini et al. 2009; Thines et al. 2007; Staswick & Tiryaki. 2004). SCF^{COI1} then tags the captive JAZ proteins, which are negative regulators of jasmonate signaling, with the polypeptide

ubiquitin, damning them to rapid destruction. (Thines et al. 2007; Chung et al. 2009).

Transcription factor repression in the JA pathway has subsequently been expanded on with the discovery of the adaptor protein novel interactor of JAZ (NINJA), with the JAZ ZIM domain acting as a binding site for NINJA. NINJA has been shown to facilitate the recruitment of TOPLESS (TPL) co repressors, TOPLESS related 2 (TPR2) and TPR3 (Pauwels *et al.* 2010; Kazan & Manners. 2011). The binding of NINJA to the SCF^{COI1} – JAZ complex is mediated by ethylene response factor (ERF), associated amphiphilic repression motif (EAR) (Kazan & Manners. 2011). The repression of JA related transcription factors is thought to occur as a result of the dual effect of JAZ proteins and specific co repressors such as TPL.

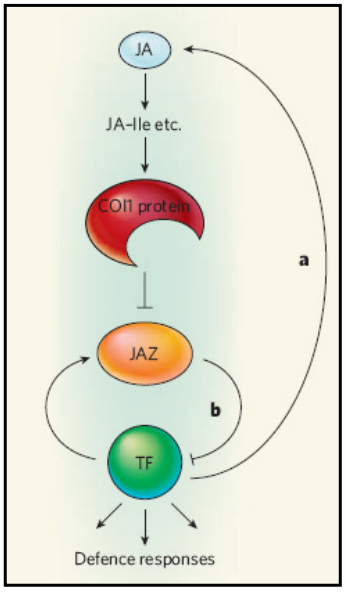


Figure 5. Primary regulatory cycles in JA signalling. Synthesis of jasmonic acid (JA) is a self-promoting feed-forward loop (a). Loop b is the newly discovered negative feedback loop involving JAZ proteins and transcription factors (TF) such as MYC2 **(**Farmer EE. 2007).

Due to the importance and function of the JA pathway, JAZ proteins have since been found to be targets for pathogen effectors (Gimenez-Ibanez et al. 2014). In a recent study the *P. syringae* effector protein HopX1 has been shown to decrease the accumulation of all JAZ proteins, by targeting JAZ proteins for degradation through their conserved central ZIM domain. The study also shows HopX1 encodes a cysteine protease causing JAZ degradation in a COI1-independent manner (Gimenez-Ibanez et al. 2014).

This results in JA dependent gene expression and the repression of SA induced markers. This is an interesting finding as SA accumulation is required for an effective HR and SAR (Zheng et al. 2012; Despres et al. 2003; Rasmussen et

al. 1991), compromising the immune response of the host. In addition Gimenez-Ibanez (2014) also showed that ectopic expression of HopX1 in *A. thaliana* complemented the growth of a COR-deficient *P. syringae* pv. tomato DC3000 strain. The findings suggest "HopX1 illustrates a paradigm of an alternative evolutionary solution to COR with a similar physiological outcome" (Gimenez-Ibanez et al. 2014). Indicating that COR and HopX1 carry out a similar function, supporting the hypothesis that COR, (as a JA-isoleucine structural mimic), plays a role in inducing the JA signalling pathway.

JAZ proteins have been shown to be a target for invading pathogens via effector proteins and by the phytotoxin coronatine (Melotto et al. 2008; Gimenez-Ibanez et al. 2014). In this project I continue to examine the effect of specific JAZ protein(s) deficiency on the plant susceptibility and resistance to plant pathogens using the model pathogen *P. syringae*. In order to assess the role of JAZ proteins in plant resistance or susceptibility to pathogen attack further JAZ knock out lines are required to assess their effect on pathogenicity.

In addition, I continue to investigate reports of high coronatine levels in a particular JAZ knock out *A. thailana* line the *jaz* 5/10 knock out line (M.Grant laboratory, unpublished data). Using the technique Q-PCR I intend to measure gene experession levels of coronatine associated genes in *P. syringae* during the infection of the *jaz* 5/10 double knock out mutant line.

2: Aims and Objectives

The overall goal of this investigation is to uncover the roles of JAZ proteins in the innate immune response to pathogen attack. As previously mentioned in the introduction to jasmonate signalling, JA signalling is an intrinsic aspect of plant responses to a medley of stresses. Importantly in this case pathogen attack. It has been shown that JAZ proteins play a role in pathogen susceptibility and can in fact be targets of pathogen effector proteins (Gimenez-Ibanez et al. 2014).

I plan to assess the role of JAZ proteins in disease susceptibility and or resistance. Furthermore this study plans to expand upon unpublished data that suggests coronatine specific susceptibility in the *jaz* 5/10 mutant *A. thaliana* line.

2.1: Aims

1. To expand upon the existing catalogue of JAZ mutant *A. thaliana* lines.
2. To assess the susceptibility or resistance of all available JAZ mutant *A. thaliana* lines.
3. To investigate the relationship between coronatine and susceptibility in the *jaz* 5/10 mutant *A. thaliana* line.

2.2: Objectives

1. To cross breed existing JAZ mutant *A. thaliana* lines.
2. To genotype the f1 generation of crossbred existing JAZ mutant lines to isolate hybrid plants that have new combinations of JAZ mutations.
3. To inoculate all available (including newly isolated) JAZ mutant lines with the pathogen *P. syringae* DC3000 and assess the levels of bacterial growth.
4. To inoculate all available (including newly isolated) JAZ mutant lines with the pathogen *P. syringae* DC3000 and assess leaf lesion development and necrosis.
5. To assess the susceptibility of the *A. thaliana jaz* 5/10 insertional mutant line to *P. syringae* DC3000 and *P. syringae* DC3000 coronatine impaired mutant (COR double).
6. To assess coronatine associated gene expression in *P. syringae* DC3000 during the infection of the *A. thaliana jaz* 5/10 insertional mutant line comparatively to wild type *A. thaliana*.

3: Materials and Methods

3.1: Plant material and growth conditions

A. thaliana KO mutant line seeds were obtained from The Nottingham Arabidopsis Stock Centre (NASC) (see table 1). The seeds were sown in a combination of 2/3 John Innes potting compost No.2 and 1/3 Sinclair vermiculite. Pots containing the sown seeds were covered with foil and put into dark conditions at 4^{o}C for 2 days to vernalise. After this the pots were transferred to a controlled environment chamber (10hr light, 100-125 µEinstein/m2/sec at 22^{o}C day and 20^{o}C night). 10 days later seedlings were pricked out into 5x5cm sectioned disposable plastic trays, each tray containing 24 pots per tray. Transparent plastic domes were placed on top of each tray to increase humidity. Plants were left to grow in the growth room for 4-5 weeks before being used in experiments (deTorres-Zabala *et al.* 2003).

JAZ KO mutants		NASC number
Single	*jaz* 1 (At1g19180)	N511957
	jaz 2 (At1g74950)	N657356
	jaz 3 (At3g17860)	N660625
	jaz 5 (At1g17380)	N553775
	jaz 6 (At1g72450)	N878214
	jaz 7 (At2g34600)	N849196
	jaz 10 (At5g13220)	N872819
Double	*jaz* 1/3	N/A
	jaz 3/5	N/A
	jaz 2/7	N/A
	jaz 6/7	N/A
	jaz 5/10	N/A
Triple	*jaz* 3/5/10	N/A

Table 1. Details of sourced plant material. All single mutants were sourced from the NASC. Double and treble mutants were sourced from the M.Grant laboratory.

3.2: Pathogenic materials

Two strains of *P. syringae* were used. Firstly *P. syringae* DC3000 containing an empty vector pVSP61 and a coronatine defective strain of *P. syringae* DC3000 (Δcfa6Δcma) (M.Grant laboratory). Both strains were grown on solidified King's B media (KB) (King *et al.*1954), containing antibiotics for selection. DC3000 pVSP61 was selected for using the antibiotics rifampicin (Sigma-Aldrich) and kanamycin (Sigma-Aldrich) at the respective concentrations 50 µg/ml and 25 µg/ml. DC3000 Δcfa6Δcma was selected for using rifampicin, kanamycin and spectinomycin (Sigma-Aldrich) at the respective concentrations 50 µg/ml, 25 µg/ml and 25 µg/ml (deTorres-Zabala *et al.* 2003).

3.3: *P. syringae* inoculation

P. syringae bacteria were grown in liquid KB media containing antibiotics for selection (see 3.2), overnight in a 28^{o}C shaker. The bacteria were then washed and resuspended in 10 mM $MgCl_2$. Cell density was then measured and adjusted using a spectrophotometer to OD_{600} 0.2 (≈ 1 x 10^8 colony forming units (cfu) ml). Density was then adjusted further to OD_{600} 0.02 for spray inoculations and OD_{600} 0.002 for syringe inoculations of *A. thaliana*. The wild type Colombia (col-0) *A. thaliana* was used as a control in all experiments (deTorres-Zabala *et al.* 2003).

3.3.1: Leaf inoculation

Four fully developed and undamaged leaves were selected for inoculation in each *A. thaliana* plant. Each leaf was symmetrically and delicately nicked on either side of the central vascular vein on the abaxial surface. To perform the

infiltration a 1 ml needleless syringe containing OD_{600} 0.002 *P. syringae* in 10 mM $MgCl_2$ was placed upon the nicked regions and the bacterial suspension was delicately injected into the whole leaf tissue. Excess bacterial solution was then removed from the leaf by gently blotting with a paper towel. *P. syringae* infected plants were then placed back into the growth room for a period of 2-5 days, depending on the experiment (de Torres-Zabala. 2003).

3.3.2: Spray inoculation

Undamaged, evenly grown *A. thaliana* plants were selected for spray inoculation. The spray solution consisted of OD_{600} 0.02 *P. syringae* in 10 mM $MgCl_2$ and 0.02% surfactant (silwet). Spraying took place using a spray bottle from a range of between 20-30 cm, plants were uniformly orientated to ensure even coverage of the spray solution. The process was complete when the upper epidermis of each plant was fully and evenly coated with the spray solution (Katagiri et al.2002). Phenotypes were then qualitatively accessed over a period of 2-5 days, depending on the experiment.

3.4: Bacterial growth counts and data analysis

Four days post infection (DPI) plant leaf tissue was used to assess the levels of bacterial growth. Using a no2 5 mm diameter disc borer, a leaf disc was removed from 3 different leaves. The 3 leaves were then pooled and homogenised in 1 ml of 10 mM $MgCl_2$. The leaf discs were homogenised in a 2 ml microcentrifuge tube containing a metallic ball using a tissue lyser (Qiagen). Then 4 10X serial dilutions were made from the homogenised tissue, this was achieved by adding 100 µl of the lysate to 900 µl of 10 mM $MgCl_2$, this was then

repeated 3 more times. Bacterial growth for each plant sample was assessed by using 4-6 replicates (deTorres-Zabala *et al.* 2003).

The four serial dilutions were then plated on to individual solid KB media plates containing the relevant antibiotics for selection. For each dilution 6X 10 µl aliquots were added to the plate. All plates were then left to dry until the droplets were no longer visible. The plates were then placed inside a 28^{o}C incubator to grow for approximately 2 days (deTorres-Zabala *et al.* 2003).

Bacterial colonies of the 4 replicates were then counted at the appropriate dilutions. The average bacterial growth for each replicate was approximated and the standard deviation was then plotted. To assess significant changes between the different samples the standard deviation was then subject to single factor analysis of variance (ANOVA).

3.5: Generation of new JAZ mutant lines

Existing JAZ mutant *A. thaliana* lines (M.Grant laboratory, unpublished data) were selectively cross-pollinated by entwining two selected samples. Seeds from these samples were then harvested in paper bags, dried in a 32^{O}c incubator for 3 days. 24 to 96 plants were then grown as described in chapter 3.1 for genotyping (M.Grant laboratory).

3.6: Genotyping JAZ knock-out insertional mutant lines

3.6.1: DNA extraction protocol

Clean scissors were used to cut one leaf from each sample to be screened; the leaf was placed into a 1.5 ml microcentrifuge tube. The harvested leaves were then homogenised using a plastic pestle in 500 µl of shorty buffer (0.2 M Tris-

HCL pH9, 0.4 M LiCl, 25 mM EDTA, 1% SDS; Arabidopsis Facility Centre, University of Wisconsin Biotechnology Centre). 500 µl of phenol chloroform was then added to the samples, each sample was then vortexed and spun at room temperature for 5 minutes at maximum speed in a microcentrifgue. Then, 450 µl of the aqueous phase was delicately removed using a pipette and placed into a new 1.5 ml microcentrifuge tube. The DNA was then precipitated by adding 450 µl of isopropanol. The samples were uniformly mixed by inversion. Samples were then spun for a further 10 minutes at room temperature at maximum speed in a microcentrifuge. After this all liquid was decanted on to paper towels. The remaining visible precipitated DNA pellets were washed using 250 µl of 70% ethanol, the samples were uniformly inverted, then spun for a further 10 minutes as previously described. The liquid was then removed by pipette and remaining liquid was decanted on to paper towels and allowed to air dry for 15-20 minutes. Lastly the precipitated DNA was resuspended in 100 µl of nuclease-free water. Samples were stored a -20^{O}c, 2 µl of each sample was used for all PCR reactions (Arabidopsis Facility Centre, University of Wisconsin Biotechnology Centre).

3.6.2: Polymerase chain reaction (PCR)

The reagents for a 30 µl PCR were as follows: -

10XPCR Buffer	3µl
MgSO4 50 mM	0.9 µl (1.5 mM final vol)
dNTP's 2.5 mM	2.4 µl (200 µM final vol)
Primer F 10 µM	1.5 µl (0.5 µM final vol)
Primer R 10 µM	1.5 µl (0.5 µM final vol)
H2O	21.47 µl
Taq Polymerase	0.23 µl
Sample DNA	2 µl

The running conditions for the PCR was as follows: -

Initial denaturation: 95^0C for 1min 50sec

Denaturation: 94^0C for 30sec

Annealing X^0C for 1 min

Extension 720C for X

34 cycles

Final extension 72^0C for 5 min

X is variable based on the particular primers used in the PCR.

Table 2 details the primers used for screening each harvested sample. Each sample was screened for the presence of wild type and mutant genes. The progeny of desired homozygous mutant samples that were identified, were catalogued and kept for future use.

JAZ Gene	Forward Primer	Reverse Primer	Annealing Temp (°C)	Expected Band Size (Bp)
JAZ 5 wild type	GCTGTTGTTGTTCCTTTGTTAGG	CTTGTACTCTTCCATTTTACGCG	58	1058
jaz 5 mutant	TGGTTCACGTAGTGGGCCATCG	CTTGTACTCTTCCATTTTACGCG	58	749
JAZ 10 wild type	TACTCCGAGGGAAGATCAGC	CTTGGAAAACTGAAACACTTCC	58	245
jaz 10 mutant	TAGCATCTGAATTTCATAACCAACCTCGATACAC	CTTGGAAAACTGAAACACTTCC	58	420
JAZ 7 wild type	AAAAATGAATTGTCTCCGCG	TTCTCGGTTTACCCATTTTAGG	52	1235
jaz 7 mutant	AACGTCCGCAATGTGTTATTAAGTTGTC	TTCTCGGTTTACCCATTTTAGG	52	884
JAZ 2 wild type	AAACAAAATTGTAACTGGCTTGG	CTAGTTAAAAATGCATGCGATGG	52.5	1183
jaz 2 mutant	TGGTTCACGTAGTGGGCCATCG	CTAGTTAAAAATGCATGCGATGG	52.5	890
JAZ 6 wild type	ACTATAGCGTTTGCAAATGCC	TTAGAACAGAAATTGCAAACCG	52	1207
jaz 6 mutant	TAGCATCTGAATTTCATAACCAACCTCGATACAC	TTAGAACAGAAATTGCAAACCG	52	889

Table 2. Details of primers used for genotyping the F1 generation of JAZ mutant crosses.

3.6.3: Gel electrophoresis

PCR samples were loaded on a 1.2% agarose gel, containing 2.0 µg/ml ethidium bromide. Gels were run in a gel tank containing 1X TAE buffer (40 mM Tris, 2.2 mM Na_2EDTA). 0.2 µg of Fermentas 1kb DNA ladder was used as a molecular weight marker. The PCR products were visualized using a UV transilluminator and the image was then photographed. The PCR illustrated the findings by means of a positive/negative screen, where the PCR was used to screen for the presence or absence of wild type and mutant bands. Primers

were used to screen for wild type bands as well as insertion specific primers to screen for the presence of mutations (see table 2). Samples containing wild type and mutant bands showed heterozygous samples.

3.7: Plant hormone extraction

Using clean scissors selected leaf tissue was cut from the plant, the tissue was then immediately wrapped in foil and snap frozen in liquid nitrogen. Each sample consisted of 3 replicates and 4 leaves were harvested per replicate. The samples were then freeze-dried for 3 days. The dried leaf tissue was then transferred in to a 2 ml microcentrifuge tube. A metallic ball was then added to the sample and the tissue was then homogenised in a Qiagen tissue lyser. 10 mg of each was then weighted using a precision balance and was transferred in to a new 1.5 ml microcentrifuge tube. 400 µl of extraction solution plus internal standards was then added to each 10 mg sample. 400 µl of the extraction solution and internal standards was used as a control, 1 control is required for every 25 samples.

Preparation of extraction solution plus internal standards was as follows. Extraction solution: 10% methanol and 1% acetic acid in ddH2O (all reagents must be HPLC quality reagents). Each sample required 395 µl of extraction solution, 2 µl of JA (24 µM), 1 µl of SA (100 µM) and 2 ul of ABA (10 µM). Samples were then vortexed thoroughly until all powdered leaf tissue was suspended in solution. Samples were then left on ice for 30 minutes, being vortexed every 10 minutes. After, samples were then spun for 10 minutes at max speed at 4^{o}C in a microcentrifuge. The supernatant was then carefully removed with a pipette and transferred to a clean 1.5 ml microcentrifuge tube. 200 µl of sample was then transferred into a glass vial for LC-MS analysis.

3.8: Quantification of bacterial gene expression

3.8.1: RNA extraction protocol

Using clean scissors selected leaf tissue was cut from the plant, the tissue was immediately wrapped in foil and snap frozen in liquid nitrogen. The frozen leaf tissue was then placed in to a mortar pre-cooled with liquid nitrogen and grinded into a fine powder, ensuring the tissue never thawed. 100 µl of Z6 buffer (per extracted leaf) from an RNA isolation kit (Roche) was added to the tissue and then mixed thoroughly using the pestle. The mixture was then transferred to a 1.5 ml microcentrifuge tube. A further 100 µl of Z6 buffer was added to the mortar, mixed with the pestle and was pooled with the previously transferred Z6 buffer-leaf extract mixture. 1 volume of Phenol/chloroform was then added to the sample and vortexed vigorously. The sample was then spun for 5 minutes at max speed in a microcentrifuge at 4^{o}c. The aqueous phase was then passed to a new 1.5 ml microcentrifuge and a 1/20 volume of acetic acid (1 M) and a 0.7 volume of ethanol (100%) were added to the sample, which was then mixed by vortex and kept on ice for 30 minutes. After, the sample was spun for 20 minutes at max speed in a microcentrifuge at 4^{o}c and the liquid was decanted on to paper towels.

The visibly precipitated nucleic acid pellet was then washed with 500 µl of 70% ethanol; the sample was mixed by inversion and spun for 5 minutes at max speed at 4^{o}C in a microcentrifuge. The supernatant was then removed using a pipette and any remaining liquid was decanted on to paper towel, the pellet was then left to air dry for 15-20 minutes. The RNA pellet was then resuspended in 100 µl of nuclease-free water by vortexing. The sample was then incubated for

10 minutes at 65°C and vortexed again until the pellet was completely resuspended. Samples were then stored at –80°C (Roche, RNA isolation kit protocol).

3.8.2: cDNA synthesis protocol

RNA samples were diluted to a concentration of 0.5 µg/µl. A master mix was created comprising of 1 µl 10X DNAse buffer, 4.5 µl RNase free water and 1.5 µl DNase per sample for a 10 µl reaction. 7 µl of the master mix was then added to a 0.2 ml PCR tube along with 1.5 µg of sample RNA, the mixture was then incubated at 37°C for 30 minutes, after which 1 µl of stop solution was added.

For the reverse transcription, A new master mix was created consisting of 0.5 µl of Oligo dT (100 µM), 4 µl of dNTP's (2.5 mM) and 1.5 µl RNAse free water per sample. 6 µl of the master mix was then added to a new 0.2 ml PCR tube followed by the addition of 7 µl of the sample previously described, giving a total volume of 13 µl. The mixture was then incubated at 65°C for 5 minutes and then placed immediately on ice for 1 minute.

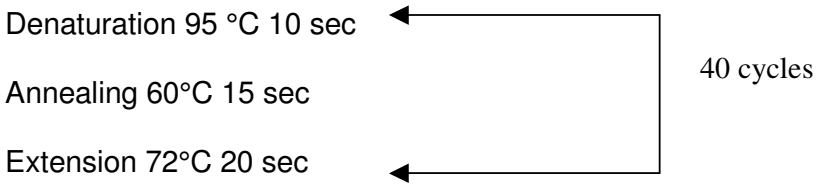
A final master mix was then prepared consisting of 4 µl of 5X RT buffer, 1 µl RNAse free water, 1 µl of 0.1 M DTT and 1 µl of Superscript III reverse transcriptase per sample. 7 µl of this mixture was then added to each sample giving a total reaction volume of 20 µl.

The samples were then incubated for 60 minutes at 50°C, then at 70°C for 15 minutes. The samples were then immediately placed on ice or stored at –20°C (Qiagen, reverse transcription kit protocol). Reagents were provided from a reverse transcription kit (Qiagen).

3.8.3 Quantitative-Polymerise chain reaction (Q-PCR)

cDNA samples were diluted to 5 ng/µl. A master mix was assembled using reagents from a universal SYBR green Q-PCR kit (Sigma-Aldrich) comprising of 10 µl of 2X Q-PCR mix, 0.9 µl 10 µM forward primer, 0.9 µl 10 µM reverse primer and 7.2 µl of PCR grade water, per sample. 19 µl of the master mix was added to a 0.2 ml PCR tube, followed by 1 µl of 5 ng/µl cDNA sample, giving a 20 µl reaction volume. Samples were then run on a 6000 series Rotor gene Q-PCR machine. The PCR conditions were as follows:

Initial denaturation 95 °C 10 min



Serial dilutions of known concentrations of actin were used as standards. Four dilutions were included with each run, at the following concentrations: 1000 fg/µl, 100 fg/µl, 10 fg/µl and 1 fg/µl. In addition the bacterial house keeping gene *gap1* was included with all samples to assess general gene expression in the run. The results were then analysed using rotor gene 6000 series software and using the known actin standards the relative concentrations of each sample were calculated and compared. The levels of *gap1* gene expression were included as error bars in generated bar graphs comparing relative RNA concentrations.

Chapter 4: Results

4.1: Generation of Jasmonate-ZIM domain (JAZ) gene mutant transgenic lines.

JAZ genes are hypothesised and have shown to be a direct or indirect target for degradation by invading pathogens via effector proteins to facilitate pathogenesis.

In order to establish a role for JAZ proteins in disease susceptibility, transfer DNA (T-DNA) was randomly inserted into the *A. thaliana* genome and through intensive screening single JAZ insertional mutants were identified from the mutant population.

As previously discussed; JAZ proteins bind transcription factors in the JA signalling pathway following JAZ protein degradation the bound transcription factor is released, allowing for the up regulation of genes associated with the JA signalling pathway. Furthermore, in the broad spectrum of genes up regulated are genes encoding for JAZ proteins themselves, creating a negative feedback loop stopping constitutive expression.

It is hypothesised that transcription factors bound by JAZ repressors can initiate the up regulation of multiple JAZ repressors. Therefore to assess the role of JAZ proteins in pathogen susceptibility thoroughly transgenic lines with not only single but also multiple JAZ mutations are required.

This work was underway in the Grant laboratory prior to my research. To build upon this study I expanded the library of double and treble JAZ mutated transgenic lines.

4.1.1: Identification of JAZ double insertional mutant transgenic lines, *jaz 7/5* and *jaz 7/10*.

Jaz 7 and *jaz 5/10* insertional mutant transgenic lines were crossbred using previously described methods. The F1 generations were then genotyped for homozygous *jaz 7/5*, *7/10* and *jaz 7/5/10* KO mutations.

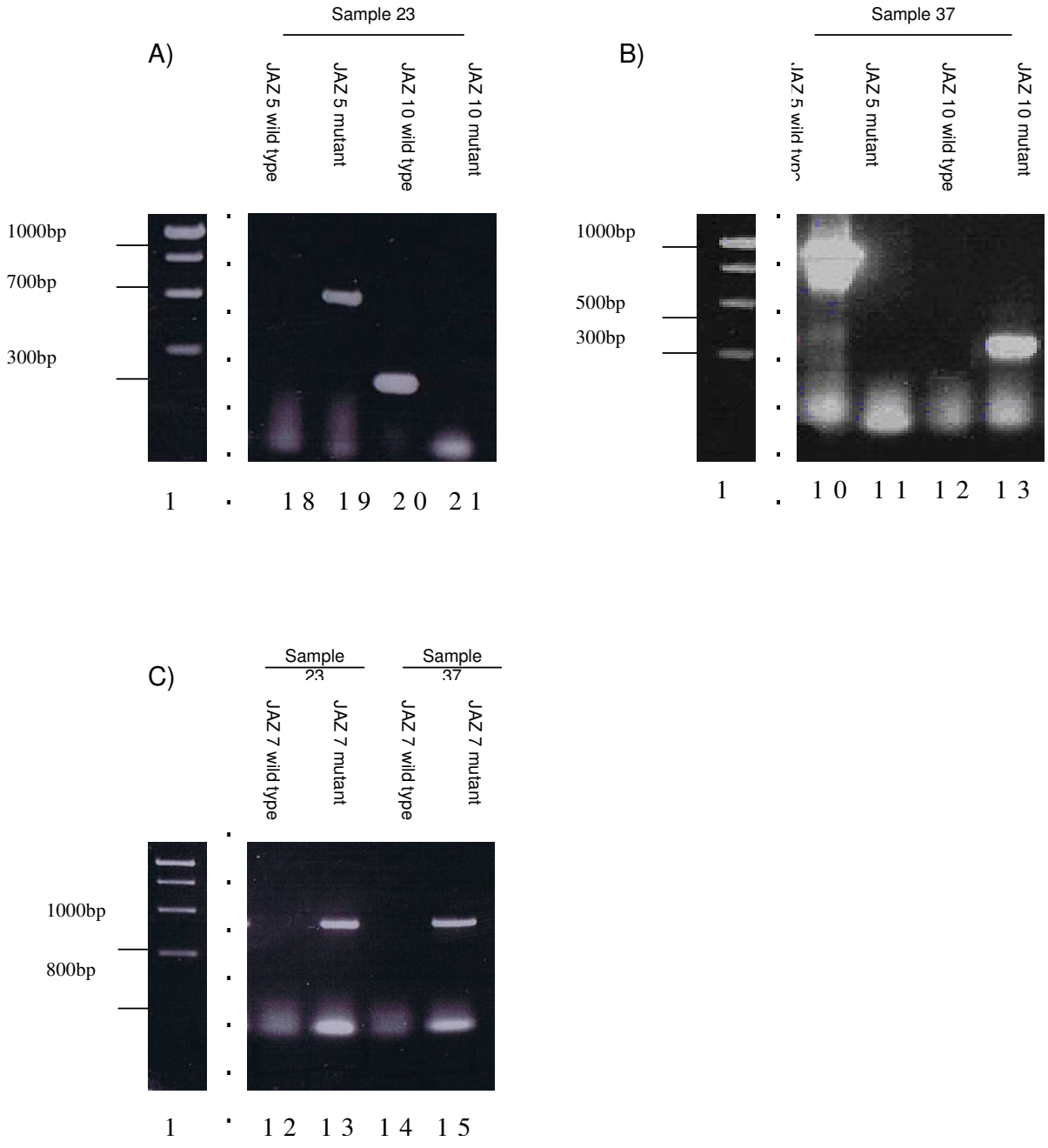


Figure 6. Results of genotyping the F1 generation from a *jaz 7* and *jaz 5/10* mutant line cross via agarose gel electrophoresis. A) & C) *A. thaliana* sample 23 genotyped as a *jaz 7/5* insertional double mutant, shown by the amplification of T-DNA insert. B) & C) *A. thaliana* sample 27 genotyped as a *jaz 7*/10 insertional double mutant, shown by the amplification of inserted T-DNA.

F1 generation mutants were genotyped for the presence of wild type and insertionally mutated *JAZ* genes. The DNA of each plant sample was genotyped by means of PCR. The extracted DNA from each *A. thaliana* sample was used to run two PCRs per *JAZ* gene.

One PCR was used to screen wild type *JAZ* genes using primers complementary to the wild type *JAZ* gene sequence. The second PCR was used to screen for insertional mutant JAZ genes, containing primers complementary to sequence only found in insertional mutants. Details of the primers and annealing temperatures used for this PCR are detailed in table 2.

Figure 6 shows the successful identification of two double *JAZ* gene mutated lines (*jaz 7/5* & *7/10*) through agarose gel electrophoresis; the findings of figure 6 are condensed into a summary table (listed below).

Sample No	Wild type *JAZ 5* gene present?	Mutant *jaz 5* gene present?	Wild type *JAZ 10* gene present?	Mutant *jaz 10* gene present?	Wild type *JAZ 7* gene present?	Mutant *jaz 7* gene present?	Summary
23	No, fig 6A) lane18	Yes, fig 6A) lane 19	Yes, fig 6A) lane 20	No, fig 6A) lane 21	No, fig 6C) lane 12	Yes, fig 6C) lane 13	Homozygous *jaz 7/5* insertional mutant
37	Yes, fig 6B) lane 10	No, fig 6B) lane 11	No, fig 6B) lane 12	Yes, fig 6B) lane 13	No, fig 6C) lane 14	Yes, fig 6C) lane 15	Homozygous *jaz 7/10* insertional mutant

Table 3. Summary table illustrating the findings from genotyping *jaz 7* and *jaz 5/10* mutant line cross. The table describes the identification of A. *thaliana* sample 23 as a *jaz 7/5* double insertional mutant and sample 37 as a *jaz 7/10* double insertional mutant.

4.1.2: Identification of a JAZ double and treble insertional mutant transgenic line, *jaz 2/5* and *jaz 2/5/10*.

Two further transgenic lines were identified genotyping the F1 generation from cross breeding two *JAZ* insertional mutant transgenic *A. thaliana* lines, the two lines crossed are, a *jaz 2* mutant line and a *jaz 5/10* mutant line.

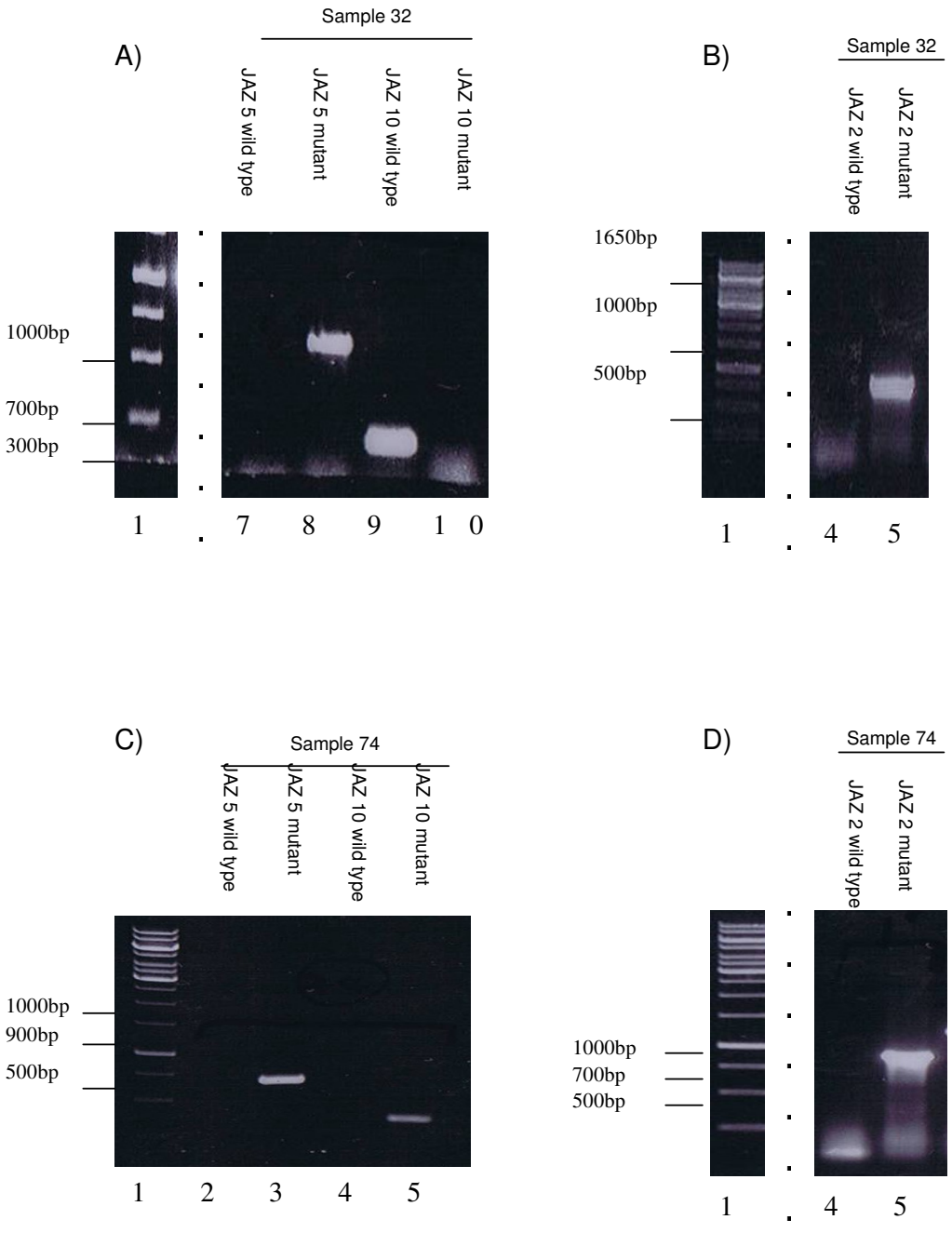


Figure 7. Results of genotyping the F1 generation from a *jaz 2* and *jaz 5/10* Mutant line cross via agarose gel electrophoresis. A) & B) *A. thaliana* sample 32 genotyped as a *jaz 2/5* insertional double mutant, shown by the amplification of T-DNA insert. C) & D) *A. thaliana* sample 74 genotyped as a *jaz 2/5* /10 insertional treble mutant, shown by the amplification of inserted T-DNA.

The findings of this screen are summarised in table 4.

Sample No	Wild type *jaz* 5 gene present?	Mutant *jaz* 5 gene present?	Wild type *JAZ* 10 gene present?	Mutant *jaz* 10 gene present?	Wild type *JAZ* 2 gene present?	Mutant *jaz* 2 gene present?	Summary
32	No, fig 8A) lane 7	Yes, fig 8A) lane 8	Yes, fig 8A) lane 9	No, fig 8A) lane 10	No, fig 8B) lane 4	Yes, fig 8B) lane 5	Homozygous *jaz* 2/5 insertional mutant
74	No, fig 8C) lane 2	Yes, fig 8C) lane 3	No, fig 8C) lane 4	Yes, fig 8C) lane 5	No, fig 8D) lane 4	Yes, fig 8D) lane 5	Homozygous *jaz* 2/5/10 insertional mutant

Table 4. Summary table illustrating the findings from genotyping a *jaz 2* and *jaz 5/10* mutant line cross. *A. thaliana* sample 32 is identified a *jaz 2/5* double insertional mutant and sample 74 as a *jaz 2/5/10* treble insertional mutant.

4.1.3: Identification of the JAZ treble insertional mutant transgenic line, *jaz 6/5/10*.

Two *A. thaliana* lines, *jaz 6* and *jaz 5/10* insertional mutant transgenic lines were crossbred. The F1 generation were then genotyped for homozygous double and treble JAZ mutant samples, in this screen a *jaz 6/5/10* insertional mutant line was identified.

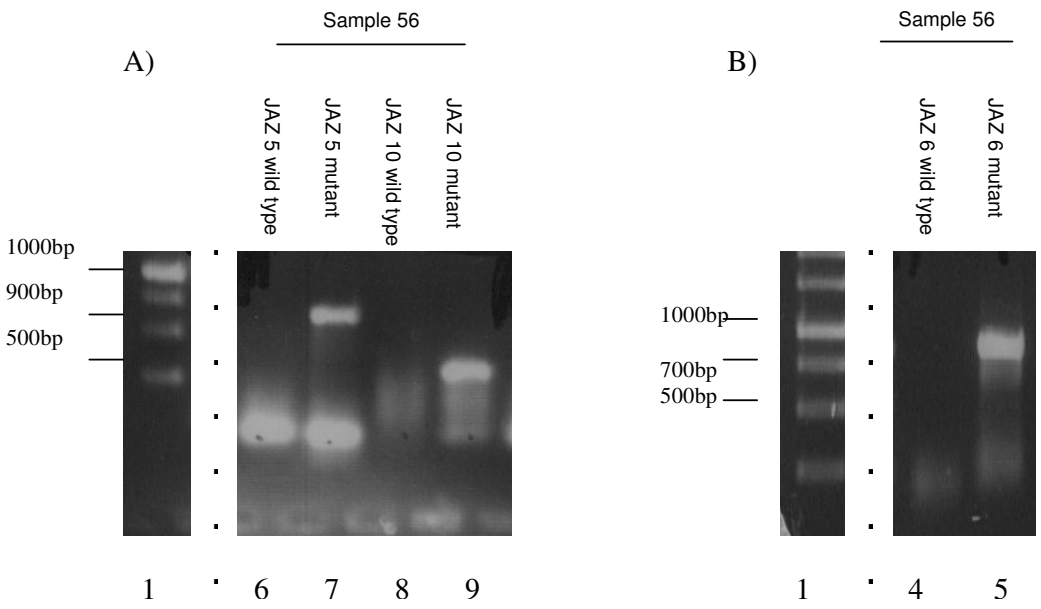


Figure 8. Results of genotyping the F1 generation from a *jaz* 6 and *jaz* 5/10 mutant line cross via agarose gel electrophoresis. A) & B) *A. thaliana* sample 56 genotyped as a *jaz 6/5/10* insertional treble mutant, shown by the amplification of inserted T-DNA.

Figure 8 shows the identification of a *jaz* 6/5/10 treble insertional mutant line. The findings of this screen are summarised in table 5.

Sample No	Wild type *JAZ* 5 gene present?	Mutant *jaz* 5 gene present?	Wild type *JAZ* 10 gene present?	Mutant *jaz* 10 gene present?	Wild type JAZ 6 gene present?	Mutant *jaz* 6 gene present?	Summary
56	No, fig 10A) lane 6	Yes, fig 10A) lane 7	No, fig 10A) lane 8	Yes, fig 10A) lane 9	No, fig 10B) lane 4	Yes, fig 10B) lane 5	Homozygous *jaz* 6/5/10 insertional mutant

Table 5. Summary table illustrating the findings from genotyping a *jaz 6* and *jaz 5/10* mutant line cross. The table describes the identification of *A. thaliana* sample 56 as a *jaz 2/5/10* treble insertional mutant.

4.2: Growth Curves of *P. syringae* in *A. thaliana* JAZ insertional mutation transgenic lines.

In order to assess the role of JAZ proteins in pathogen susceptibility and resistance, the growth of *P. syringae* was measured in leaf tissue local to the site of infection. This was carried out in all available lab strains of JAZ insertional mutants and compared to the *A. thaliana* wild type col-0.

4.2.1: *P. syringae* growth measurements in single JAZ mutant transgenic lines.

Firstly bacterial growth was measured in all available single JAZ mutant lines.

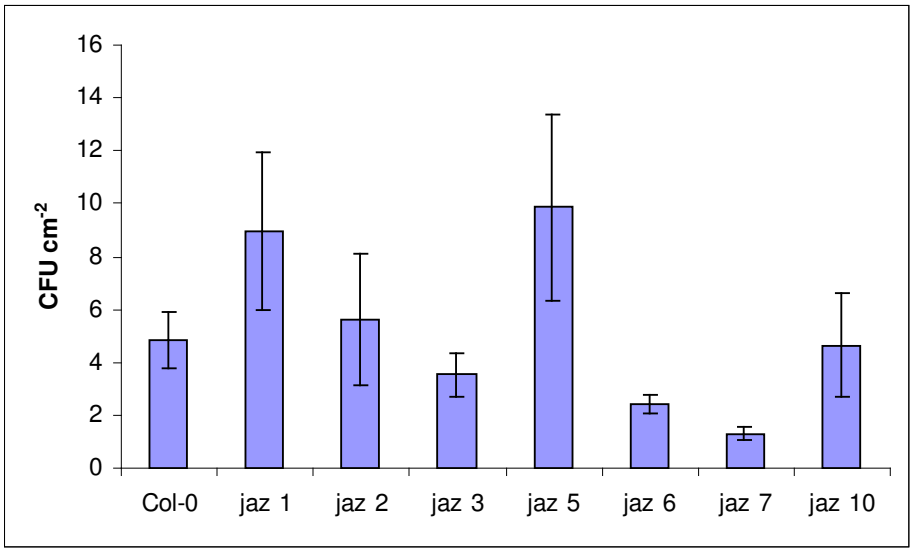


Figure 9. Bar graph of *P. syringae* DC3000 growth in *A. thaliana* single JAZ insertional mutant lines. Samples were inoculated with OD600=0.002 *P. syringae* and leaf tissue harvested 4 DPI. Error bars represent standard error.

Figure 9 shows the variations in bacteria l growth in a range of single JAZ mutants, differences in bacterial growth can be observed in single JAZ deficient mutants. Most notably *jaz 6* and *jaz 7* single mutants show decreased bacterial growth. To determine if the changes of *P. syringae* growth between JAZ single mutant samples are statistically significant between each other and the wild type, I conducted single factor analysis of variance (ANOVA) in conjunction with a *t-test*. ANOVA is a powerful analytical tool that allows the comparison of multiple means to evaluate the significance. In the case of a single factor ANOVA, the differences in bacterial growth are assessed compared to changes in JAZ protein expression, whilst taking into account the variance between all samples in a given group.

In table 6A) *P-values* and an *F-test* were generated for each single JAZ mutant compared to the wild type. In table 6B) a *P-value* and *F-test* were generated comparing the variation between all JAZ samples and the wild type together.

A)

Sample compared to wild type Col-0	*P-value*	Significant?	*F-value*	*F critical value*	Significant?
jaz 1	0.238	N	1.716	5.987	N
jaz 2	0.773	N	0.091	5.987	N
jaz 3	0.376	N	0.916	5.987	N
jaz 5	0.217	N	1.900	5.987	N
jaz 6	0.078	N	4.522	5.987	N
***jaz* 7**	**0.018**	**Y**	**10.432**	**5.987**	**Y**
jaz 10	0.940	N	0.006	5.987	N

B)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.49E+08	7	35551100	2.143813	0.077469	2.422631
Within Groups	3.98E+08	24	16583116			
Total	6.47E+08	31				

Table 6. Single factor analysis of variance (ANOVA) results on *P. syringae* growth data in *A. thaliana* single JAZ mutants. A) Variance between each JAZ single mutant and the wild type col-0 is assessed for significance B) Variance between all JAZ single mutants and the wild type are assessed to ascertain if there is any statistically significant variance between any mutant lines.

Statistically the *JAZ 7* single mutation is the only single mutation with a significant *P-value* of 0.018, in addition rejecting the null hypothesis of the *F-test* (that there is no significant variance) with an *F-value* exceeding the *F-critical* value.

4.2.2: *P. syringae* growth measurements in double JAZ mutant transgenic lines.

Bacterial growth was measured in all available double JAZ mutant lines.

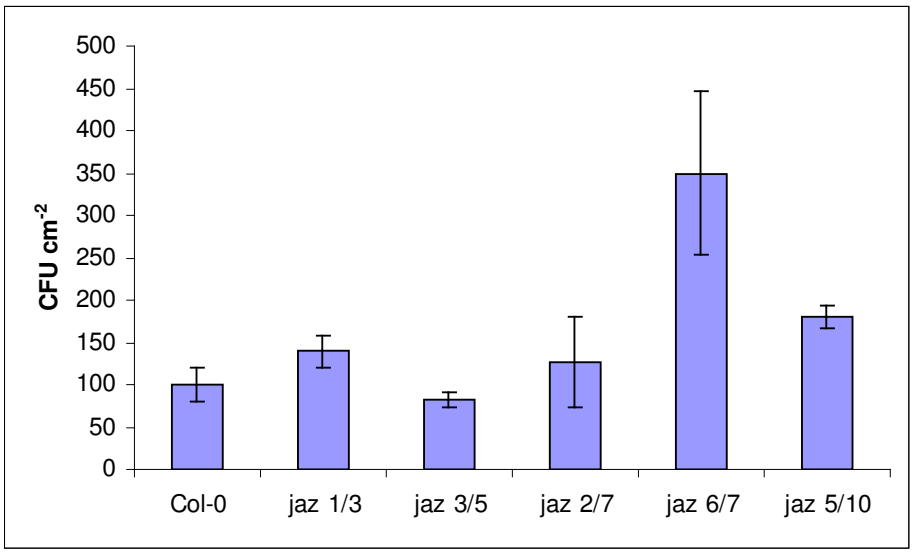


Figure 10. Bar graph of *P. syringae* DC3000 growth in double JAZ insertional mutant *A. thaliana* lines. Samples were inoculated with OD600=0.002 *P. syringae* and leaf tissue harvested 4 DPI. Error bars represent standard error.

In figure 10 variations in bacterial growth between JAZ mutant samples can be observed. To determine any statistical significance between double JAZ mutant *A. thaliana* lines, a single factor ANOVA was carried out on all samples. Independently comparing all JAZ mutants to the wild type and variance between all samples and the wild type being compared together.

A)

Sample compared to wild type Col-0	*P-value*	Significant?	*F-value*	*F critical value*	Significant?
jaz 1/3	0.17913	N	2.0872	4.96459	N
jaz 3/5	0.42827	N	0.6817	4.96459	N
jaz 2/7	0.65886	N	0.207	4.96459	N
***jaz* 6/7**	**0.0304**	**Y**	**6.3491**	**4.96459**	**Y**
***jaz* 5/10**	**0.0067**	**Y**	**11.601**	**4.96459**	**Y**

B)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.57E+11	4	6.43E+10	4.14124	0.010399	2.758711
Within Groups	3.88E+11	25	1.55E+10			
Total	6.45E+11	29				

Table 7. Single factor ANOVA was conducted on bacterial growth data in *A. thaliana* double JAZ mutants. A) Variance between each JAZ double mutant and the wild type col-0 is assessed for significance B) Variance between all JAZ double mutants and the wild type are assessed to ascertain if there is any statistically significant variance between any of the samples.

Table 7 shows that both the *jaz 6/7* and *jaz 5/10* double mutants have a statistically significant *P-value*. The *F-value* also exceeds the *F-critical* value supporting the hypothesis that the increases in bacterial growth observed are dependent on specific JAZ mutations.

4.2.3: *P. syringae* growth measurements in a treble JAZ mutant transgenic line.

Bacterial growth was measured in the currently, only available treble JAZ mutant line.

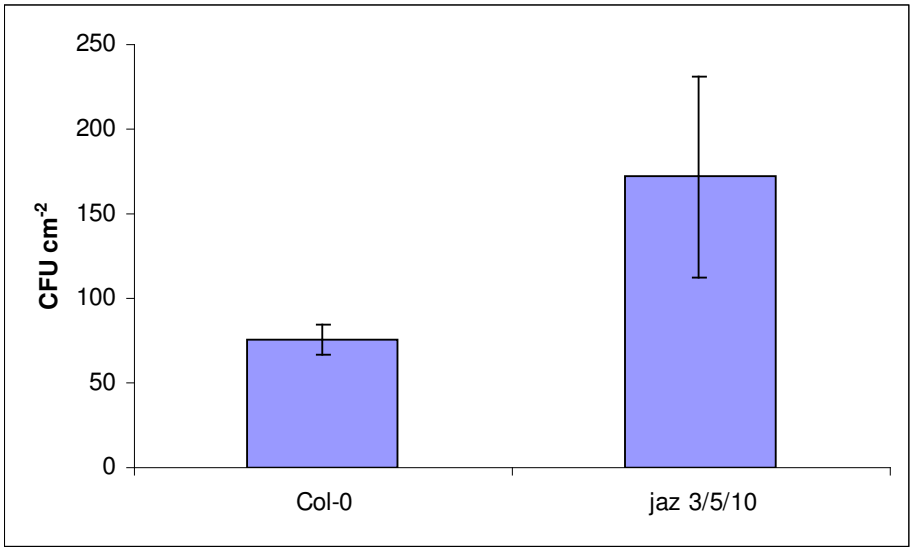


Figure 11. Bar graph of *P. syringae* DC3000 growth in the JAZ treble insertional mutant *A. thaliana* line, *jaz 3/5/10*. Samples were inoculated with OD600=0.002 *P. syringae* and leaf tissue harvested 4 DPI. Error bars represent standard error.

Figure 11 shows an increase of bacterial growth compared to the wild type. The statistical analysis of variance shown in table 8 reports no significance in either the *P-value* or the *F-test*.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.781E+12	1	2.78E+12	2.523552	0.143242	4.964591
Within Groups	1.102E+13	10	1.1E+12			
Total	1.38E+13	11				

Table 8. Single factor ANOVA was conducted on data of bacterial growth in the *A. thaliana* treble mutant *jaz 3/5/10*. Variance between the JAZ mutant and the wild type are assessed to ascertain any statistically significant variance between the sample and wild type.

4.2.4: *P. syringae* growth measurements in newly isolated JAZ mutant transgenic line.

The genotyping detailed in section 3.1 gave rise to five newly isolated mutant lines. These mutants were then inoculated with *P. syringae* to assess the potential effects of the new JAZ mutant combinations on bacterial growth.

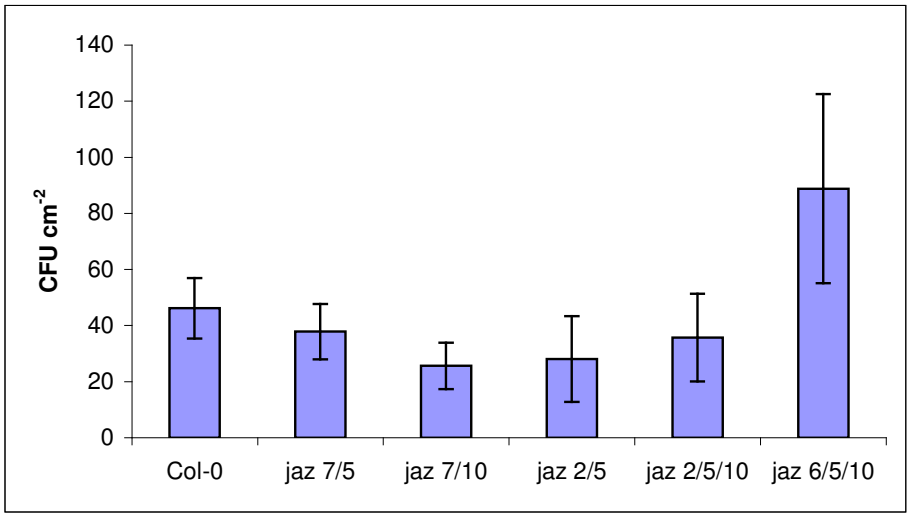


Figure 12. Bar graph of *P. syringae* DC3000 growth in newly established JAZ insertional mutant *A. thaliana* lines. Samples were inoculated with OD600=0.002 *P. syringae* and leaf tissue harvested 4 DPI. Error bars represent standard error.

Figure 12 shows the average growth of *P. syringae* in the newly isolated JAZ mutant lines, visually all the mutants do not appear to significantly differ from the wild type or among each other except for *jaz 6/5/10* showing increased growth.

Single factor analysis was carried out to determine any statistical significance between each sample and the wild type as well as any significant variation in *P. syringae* growth between any of the new JAZ mutant lines.

A)

Sample compared to wild type Col-0	*P-value*	Significant?	*F-value*	*F critical value*	Significant?
JAZ 7/5	0.581	N	0.325	4.965	N
JAZ 7/10	0.162	N	2.286	4.965	N
JAZ 2/5	0.355	N	0.942	4.965	N
JAZ 2/5/10	0.595	N	0.302	4.965	**N**
JAZ 6/5/10	0.256	N	1.451	4.965	**N**

B)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.62558E+12	5	3.2512E+11	1.7154895	0.1614681	2.533554
Within Groups	5.68555E+12	30	1.8952E+11			
Total	7.31113E+12	35				

Table 9. Single factor ANOVA was conducted on data of bacterial growth in *A. thaliana* newly established JAZ mutant lines. A) Variance between each JAZ single mutant and the wild type col-0 is assessed for significance B) Variance between all JAZ single mutants and the wild type are assessed to ascertain if there is any statistically significant variance between any of the samples.

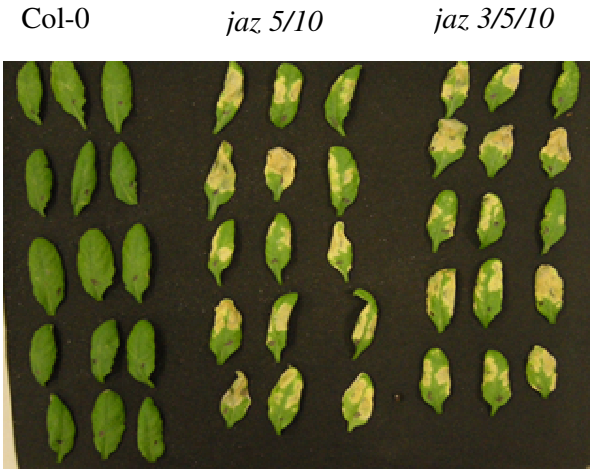
Table 9 summarises the ANOVA results, 9A) shows that bacterial growth in the newly isolated mutants doesn't significantly differ from wild type *A. thaliana*. 9B) shows no significant variation between any *A. thaliana* lines.

4.3: Phenotypes of *A. thaliana* JAZ mutant transgenic lines upon *P. syringae* DC3000 infection.

JAZ mutant *A. thaliana* lines were screened for differing phenotypes from the wild type col-0 upon *P. syringae* infection. I hypothesised that JAZ mutant lines that have significantly increased bacterial growth would exhibit stronger phenotypes.

Interestingly, the results observed only loosely supported this hypothesis. In figure 13 A) the *jaz 5/10* mutant line showed a very strong susceptible phenotype compared to the wild type, supporting the prediction based on the statistically significant increase in *P. syringae* growth in the *jaz 5/10* mutant line. Though the *jaz 3/5/10* mutant line had a comparably severe susceptible phenotype to *jaz 5/10* despite having an insignificant *P-value* and failing to show significance in the *F-test*.

A)



B)

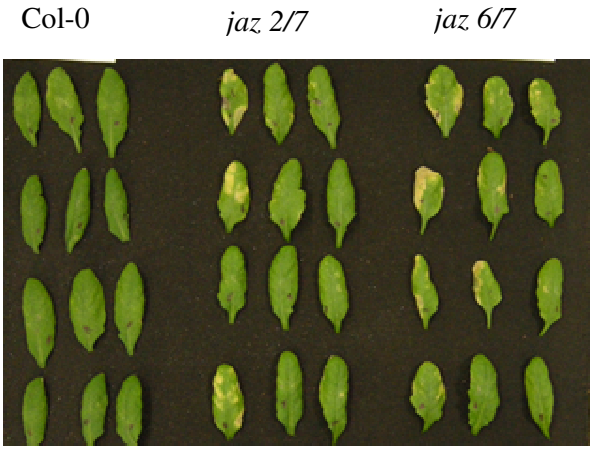


Figure 13. Phenotypes of JAZ mutant *A. thaliana* lines 4 DPI with OD600=0.02 *P. syringae DC3000* via syringe infection. A) Infected leafs extracted from *jaz 5/10* & *jaz 3/5/10* compared with Col-0. B) Infected leafs extracted from *jaz 2/7* & *jaz 6/7* compared with Col-0.

A similar observation can be seen in Fig 13 B), *jaz 6/7* showed increased susceptibility to infection compared to the wild type in addition to having statistically significant bacterial growth. However, *jaz 2/7* shows a similar phenotype to *jaz* 6/7 despite having no significant changes in bacterial growth.

4.4: Measurement of bacterial growth, observation of phenotypes and Q-PCR analysis of coronatine related gene expression upon infection of the *A. thaliana jaz 5/10* insertional mutant line with *P.syringae* DC3000.

As shown in fig 10 and fig 13 *jaz 5/10* is the most significant JAZ mutant line in terms of increased bacterial growth and phenotype strength that is available. In addition data from pervious research (see below) found that the virulence factor coronatine is found at much higher levels in the *jaz 5/10 A. thaliana* line 3 DPI with *P. syringae*. Figure 14 is an unpublished figure from Bing Zhai, illustrating a huge increase in the phytotoxin coronatine.

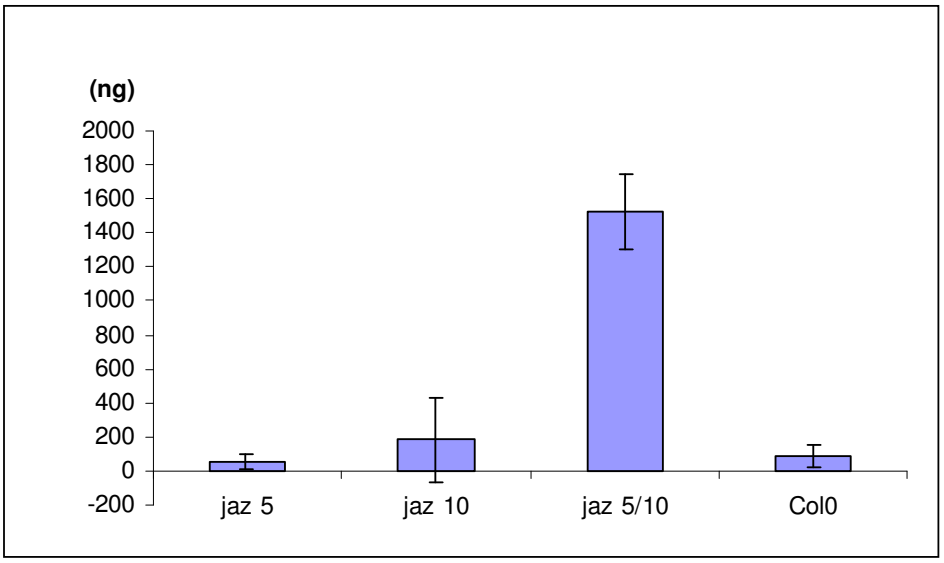


Figure 14. Comparison of quantified coronatine (ng) in the *A. thaliana* JAZ insertional mutant lines; *jaz 5*, *jaz 10* and *jaz 5/10* compared to the wild type col-0.

This section continues on from this finding, comparing levels of bacterial growth, phenotypes and coronatine associated gene expression in the *jaz 5/10 A. thaliana* mutant line upon *P. syringae* infection.

4.4.1: Growth measurements of *P. syringae* DC3000 in the *A .thaliana* insertional JAZ mutant line *jaz 5/10*.

Wild type *A. thaliana* Col-0 and the *jaz 5/10* mutant line were inoculated with *P. syringae* DC3000 and *P. syringae* COR double mutant (a strain unable to manufacture CFA and CMA, the two precursors that fuse to become coronatine), to observe the effect of coronatine on bacterial colonisation.

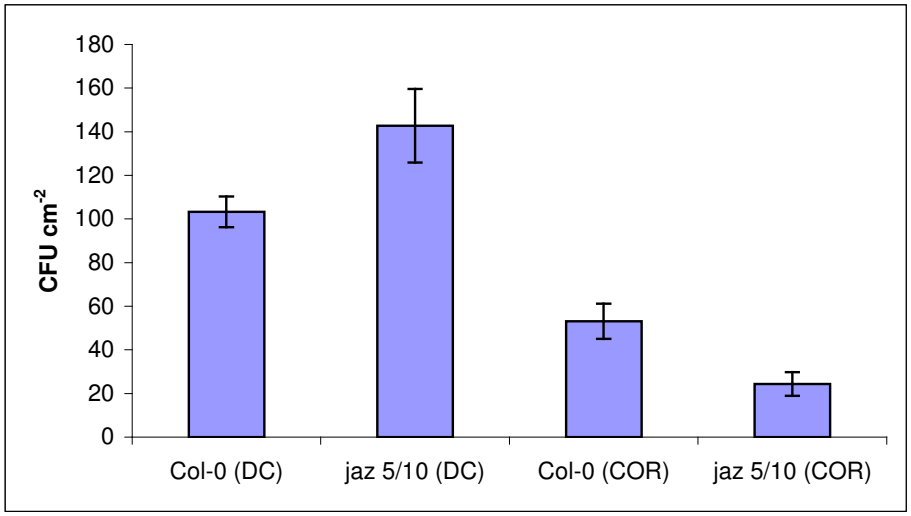


Figure 15. Bar graph of *P. syringae* DC3000 and *P. syringae* DC3000 coronatine deficient mutant growth in *A. thaliana* wild type and the jaz 5/10 mutant line. Samples were inoculated with OD600=0.002 *P. syringae* and leaf tissue was harvested 2 DPI. Error bars represent standard error.

Fig 15 shows visual reductions in bacterial growth in COR deficient *P. syringae*.

A)

Groups	*Count*	*Sum*	*Average*	*Variance*	*P-value*	*F*	*F crit*
Col-0 (DC)	6	6198333	1033056	3.021E+10	0.056011	4.670093	4.964591
JAZ 5/10 (DC)	6	8566667	1427778	1.7E+11			

B)

Groups	*Count*	*Sum*	*Average*	*Variance*	*P-value*	*F*	*F crit*
Col-0 (DC)	6	6198333	1033056	3.021E+10	0.00088	21.81128	4.964591
Col-0 (COR)	6	3183333	530555.6	3.925E+10			

C)

Groups	*Count*	*Sum*	*Average*	*Variance*	*P-value*	*F*	*F crit*
JAZ 5/10 (DC)	6	8566667	1427778	1.7E+11	5.36E-05	44.90408	4.964591
JAZ 5/10 (COR)	6	1460000	243333.3	1.749E+10			

Table 10. Single factor ANOVA was conducted on *P. syringae* DC3000 and *P. syringae* DC3000 COR double mutant bacterial growth in the *A. thaliana* double mutant *jaz 5/10*. A) assesses significant variance between *jaz 5/10* and col-0 when inoculate with *P. syringae* DC3000 B) assesses significant variance between col-0 inoculate with *P. syringae* DC3000 and the COR double mutant. C) assesses significant variance between *jaz 5/10* inoculate with *P. syringae* DC3000 and the COR double mutant.

In Table 10 A), marginal significance can be observed between the *jaz 5/10* double mutant and col-0 inoculated with *P. syringae* DC3000. 9 B) shows statistical significance between Col-0 inoculated with *P. syringae* DC3000 and the COR double mutant. In 9 C) statistical significance is observed between the JAZ mutant *jaz 5/10* inoculated with *P. syringae* DC3000 and the COR double mutant.

P. syringae DC3000 and the COR double mutant bacterial growth was calculated 3DPI.

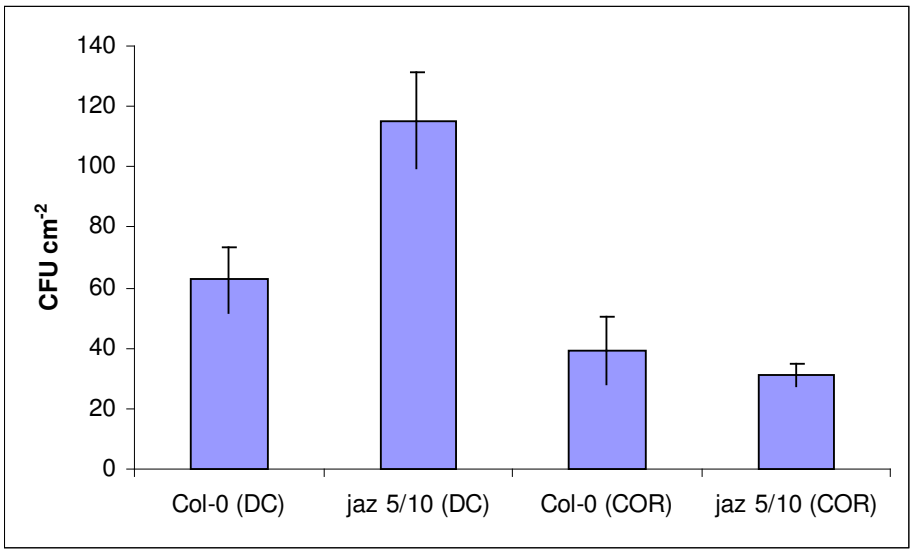


Figure 16. Bar graph of *P. syringae* DC3000 and *P. syringae* DC3000 coronatine deficient mutant growth in *A. thaliana* wild type and a *jaz 5/10* mutant lines. Samples were inoculated with OD600=0.002 *P. syringae* and leaf tissue harvested 3 DPI. Error bars represent standard error.

A)

Groups	*Count*	*Sum*	*Average*	*Variance*	*P-value*	*F*	*F crit*
Col-0 (DC)	6	3755000	625833.3	7.12E+10	0.020615	7.54124	4.964591
JAZ 5/10 (DC)	6	6916667	1152778	1.5E+11			

B)

Groups	*Count*	*Sum*	*Average*	*Variance*	*P-value*	*F*	*F crit*
Col-0 (DC)	6	3755000	625833.3	7.12E+10	0.166944	2.221599	4.964591
Col-0 (COR)	6	2353333	392222.2	7.62E+10			

C)

Groups	*Count*	*Sum*	*Average*	*Variance*	*P-value*	*F*	*F crit*
JAZ 5/10 (DC)	6	6916667	1152778	1.5E+11	0.000422	26.67944	4.964591
JAZ 5/10 (COR)	6	1870000	311666.7	9.39E+09			

Table 11. Single factor ANOVA was conducted on *P. syringae* DC3000 and *P. syringae* DC3000 COR double mutant bacterial growth in the *A. thaliana* double mutant *jaz 5/10*. A) assesses significant variance between *jaz 5/10* and col-0 when inoculate with *P. syringae* DC3000 B) assesses significant variance between col-0 inoculate with *P. syringae* DC3000 and the COR double mutant. C) assesses significant variance between *jaz 5/10* inoculate with *P. syringae* DC3000 and the COR double mutant.

Table 11A) shows statistical significance between the *jaz 5/10* double mutant and col-0 inoculated with *P. syringae* DC3000. 11B) shows no statistical significance between Col-0 inoculated with *P. syringae* DC3000 and the COR double mutant. In 11C) statistical significance is observed between the JAZ mutant *jaz 5/10* inoculated with *P. syringae* DC3000 and the COR double mutant.

4.4.2: Phenotypes of *A. thaliana* JAZ mutant lines, *jaz 5*, *jaz 10* and *jaz 5/10* upon *P. syringae* DC3000 infection.

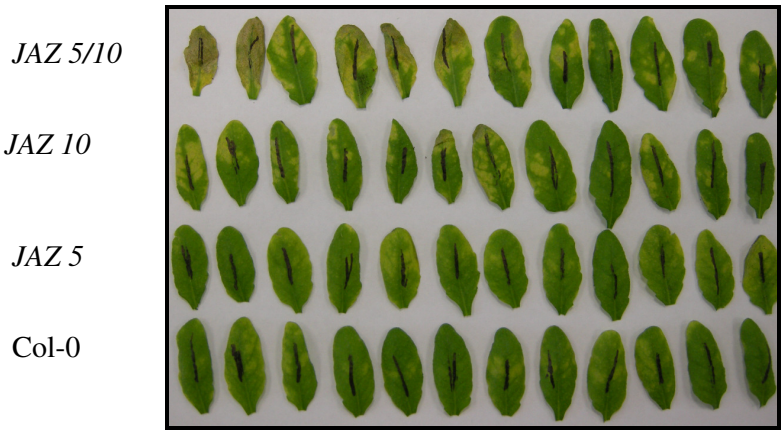


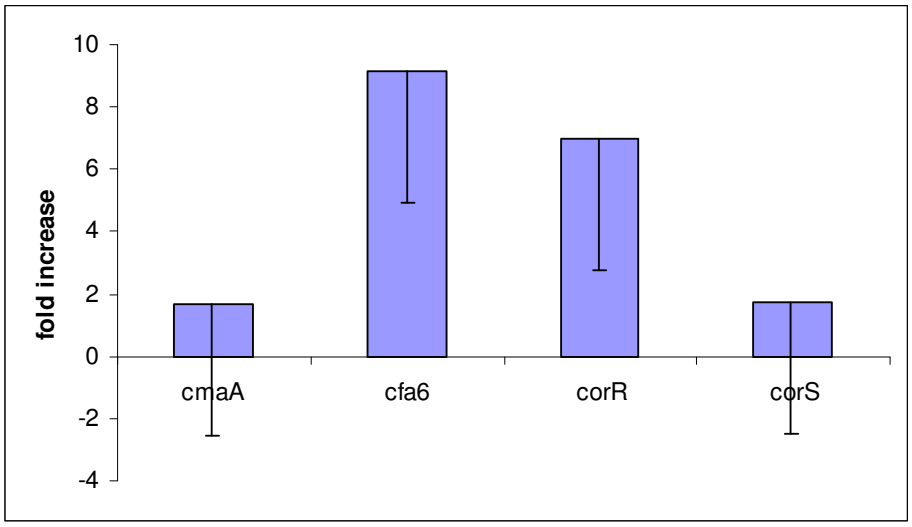
Figure 17. Phenotypes of JAZ mutant *A. thaliana* lines 3 DPI with OD600=0.02 *P. syringae DC3000*. Infected leafs extracted from *jaz 5, jaz 10 and jaz 5/10* compared with Col-0.

Fig 17 shows a strong susceptible phenotype in the *jaz 5/10* mutant line infected with *P. syringae* DC3000. A mild phenotype is also observed in the *jaz 10* mutant line, with *jaz 5* exhibiting susceptibility the same as the wild type col-0.

4.4.3: Quantitative – Polymerase chain reaction (Q-PCR), measuring gene expression of coronatine related genes; *cmaA*, *cfa6*, *corR* and *corS*.

To ascertain why greatly increased levels of coronatine are found within the *jaz 5/10* mutant line Q-PCR was performed on *P. syringae* present in infected *A. thaliana* leaf tissue. Gene expression of the following coronatine associated genes (*cmaA, cfa6, corR* and *corS*) was measured and compared between the *jaz 5/10* mutant and wild type (col-0) *A. thaliana*.

A)



B)

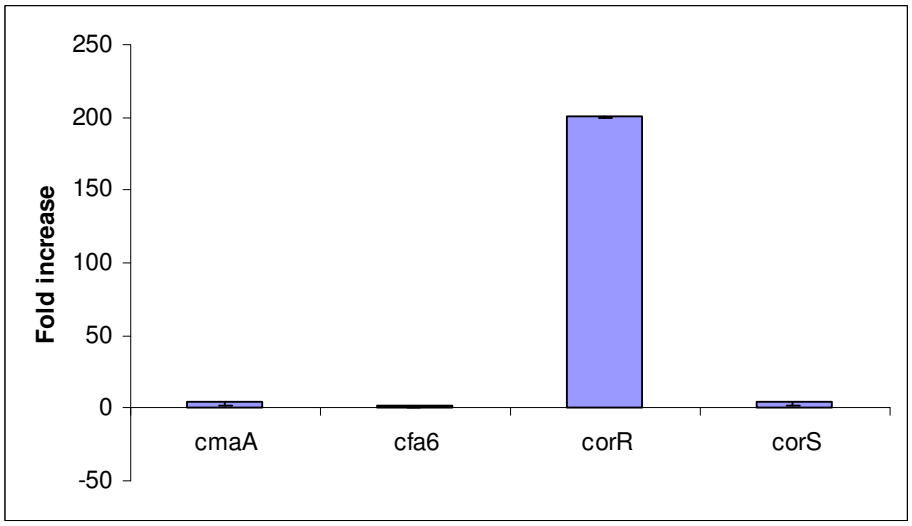


Figure 18. Fold change in coronatine associated gene expression upon *P. syringae* DC3000 inoculation in the *jaz* 5/10 mutant line compared to col-0. A) Gene expression measured 2DPI inoculation. B) Gene expression measured 3DPI inoculation. Samples were inoculated with OD600=0.02 *P. syringae DC3000*, error bars represent increase in housekeeping control gene *gap1*.

Fig 18 A) shows a four and two fold increase in *cfa6* and *corR* gene expression respectively 2DPI, after factoring the basal increase of the housekeeping gene *gap1*.

Fig 18 B) shows little measured increase in coronatine associated gene expression 3DPI except in the case of the gene *corR*. *corR* is shown to have an extremely high expression, measuring a two hundred fold increase.

Chapter 5: Discussion

5.1: Generation of Jasmonate-ZIM domain (JAZ) gene mutant transgenic lines.

In the case of the *P. syringae* pv tomato (DC3000) and *A. thaliana*, the interaction of three key plant hormones, salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) have been shown to constitute post-invasion defence responses (Kunkel et al. 2002; Derksen et al 2013; Xin & He. 2013).

Plant defence has shown to be compromised by JA and ABA as well as reducing the accumulation of SA, a pivotal part of the *A. thaliana* defence mechanism (Glazenbrook. 2005; Bari & Jones. 2008; Ton et al. 2009). The JAZ protein family transcribe repressors that constitute crucial components of the JA signalling pathway and have the potential to impact on hormone crosstalk (Chini et al. 2007). Research suggests that there is redundancy amongst JAZ proteins, although despite details of their overlapping functions there is evidence for specialization (Thines et al. 2007; Chico et al. 2008; Kazaan & Manners 2011).

To allow further and more detailed study of the role JAZ proteins play in the response to pathogen attack, new *jaz* mutant lines were created through hybridisation of existing *jaz* mutant lines. The newly isolated lines are as follows: *jaz 2/5, 7/5, 7/10, 2/5/10* and *6/5/10*. Bacterial growth and pathogenicity of *P. syringae* can be assessed in these JAZ deficient lines to elucidate a role for JAZ proteins in response to pathogen invasion.

5.2: Increased resistance to *P. syringae* infection, in the *A. thaliana jaz* 7 insertional mutant transgenic line.

Figure 9 shows the levels of bacterial growth in JAZ single mutant lines, visually some clear differences in bacterial growth can be seen to vary from wild type *A. thaliana* (Col-0). The *jaz* 7 mutant is the most notable example, showing a clear decrease in bacterial growth. The *jaz* 7 mutant line shows a three and a half fold decrease in bacterial growth as well showing no development of lesions.

This data can provides support for a concept that JAZ 7 may be involved in binding TF's that regulate genes important for a wounding response.

Yeast two hybrid studies have shown that JAZ 4 and JAZ 7 unlike the rest of JAZ proteins fail to interact with the transcription factor MYC2. While two newly identified homlogos of MYC2, MYC3 and MYC4 are shown to directly interact with all JAZ proteins including JAZ 7 (Fernández-Calvo P, et al. 2011). The details of targeted degradation of JAZ proteins during pathogen attack still remains unclear, though the unique properties of JAZ 7 only binding to the transcription factors MYC3 and MYC4 could provide a rationale for this result.

The full roles of MYC3 and MYC4 are unknown; they are both shown to associate with JAZ proteins and are among the closest known homologs of MYC2 (Schweizer F, et al. 2013; Niu et al. 2011). Either or both MYC3 and MYC4 may have roles in the up regulation of pathogen-associated genes, the insertional knockout of JAZ 7 could allow for some degree of constitutive expression of pathogen-associated genes involved in wounding response. The concept could loosely be compared to the identified phenomenon SAR (Despres et al. 2003), where a low level increase of pathogen associated genes could allow for the host to more efficiently respond to pathogen invasion and restrict the pathogens virulence. Much further work would be needed to test this

hypothesis, though this provides a potential explanation for the decrease in bacterial colonisation.

In contrast to the findings of decreased bacterial growth in the *jaz* 7 mutant line, virus-induced gene silenced JAZ lines in tomato plants have been shown to have increased bacterial growth and susceptibility to *P. Syringae* DC3000, one of which is *SIjaz7* (Ishiga Y, et al 2013).

Interestingly, susceptibility to *P. syringae* DC3000 is restored in *jaz* 7 mutants with an additional JAZ mutation. In figure 10, a statistically significant increase in bacterial growth can be seen in the *jaz* 6/7 mutant line and susceptibility is restored to a similar level as the wild type in the *jaz* 2/7 mutant line. Figure 13 shows susceptible phenotypes are exhibited in both the *jaz* 6/7 and 2/7 mutant lines. This result clearly shows that multiple changes in JAZ protein presence modulate differences in the immune response from single expression or absence of a particular JAZ protein. All bacterial growth data was performed in quadruplicate and averaged. Due to the variation between each sample the experiments will need to be repeated to ensure reproducibility.

5.3: Lesion development and leaf necrosis does not consistently correlate with changes in bacterial growth.

Figure 13 B) shows a clear increase in lesions and necrosis in the *jaz* 6/7 mutant line from the wild type, as well as a statistically significant increase in bacterial growth (table 7 A). Interestingly the *jaz* 2/7 mutant shows the same extent of susceptibility in terms of lesion development (figure 13 B) but there is no statistically significant rise in bacterial growth (table 7A).

This same result is seen in the case of the *jaz* 5/10 and the *jaz* 3/5/10 mutant lines. *jaz* 5/10 shows a strong susceptibility to *P. syringae* when observing

lesion development (figure 13 A) as well as a statistically significant increase in bacterial growth (table 7 A). However, *jaz* 3/5/10 shows a similarly strong susceptibility when observing lesion development (figure 13 A) but has no significant increase in bacterial growth (table 8). A potential explanation for the increased susceptibility to *P. syringae* could be that the absence of multiple JAZ proteins causes the release of TF's that initiate the transcription of JA-dependent genes and the repression of SA-dependent defences against the pathogen (Niki T, et al 1998). It has been shown that *P. syringae* pv. *tabaci* secretes a virulence factor HopX1 that encodes a cysteine protease which targets all JAZ proteins via the Jas motif for degradation (Gimenez-Ibanez S, et al 2014). The fact that all JAZ proteins are targeted for degradation implies that not having any JAZ repressors if more advantageous than having JAZ repressors present, therefore it is likely that having multiple JAZ KO's in one line will be more beneficial for the invading pathogen.

It is also important to note the fact that all samples that show increased bacterial growth show susceptibility when observing lesion development but not *vice versa*. Therefore significant changes in bacterial growth are not necessary for Increased pathogen virulence, indicating that leaf lesion development is a more transparent method to assess plant susceptibility. The experiments comparing bacterial growth and lesion development were performed in quadruplicate. Bacterial growth has been shown to be highly variable. To be certain that the observed correlation between bacterial growth and lesion development is inconsistent and possibly JAZ dependent, these experiments will need to be duplicated to ensure reproducibility.

5.4: Increased susceptibility to *P. syringae* infection and the phytotoxin coronatine in the double mutant *A. thaliana* transgenic line *jaz* 5/10.

Coronatine is a phytotoxin and a JA-Ile mimic, allowing the manipulation of the JA signaling pathway as to promote virulence during a *P. syringae* infection. COR binds to the COI1-JAZ receptor and induces JAZ degradation and subsequent expression of JA responsive genes therefore antagonizing SA signalling (Brooks et al. 2005; Sheard et al. 2010). COR has been shown to induce disease susceptibility in systemic tissue (Cui et al. 2005) and activate JA signalling (Melotto et al. 2008; Thines et al. 2007; Katsir et al. 2008).

Unpublished work from the M.Grant laboratory detailed greatly increased levels of coronatine via LCMS analysis in the *A. thaliana jaz 5/10* mutant line post infection with *P. syringae* as well as increased susceptibly to infection.

Table 10 and 11, show a statistically significant increased bacterial growth in JAZ 5/10 mutant lines inoculated with *P. syringae* DC3000 compared to the *P. syringae* DC3000 coronatine impaired strain. Although the effect of coronatine deficiency in *P. syringae* is not reproducibly more drastic in relation to bacterial growth in the wild type compared to the *jaz* 5/10 mutant line. However, even though coronatine deficiency doesn't appear to reproducibly affect bacterial growth more in the *jaz* 5/10 mutant line, coronatine may still be involved in the susceptible phenotype that is exhibited. Figure 18, show via Q-PCR increased coronatine associated gene expression compared to the wild type col-0. Most strikingly is a 200-fold increase in *corR* 3DPI. This result indicates cross talk between the pathogen and host. Changes in protein expression of the host may affect the secretion of effector proteins and toxins. This may potentially drive the production and delivery of tailored effector proteins to maximise the chances of

successful infiltration of the host. Coronatine associated gene expression data was generated in duplicate. The experiment requires repetition to ensure reproducibility.

5.5: Future work

The results produced from this investigation open two main channels for further research. Firstly to assess the role of the protein *jaz* 7 in disease resistance to *P. syringae* DC3000. Secondly to broadly explore the role of coronatine in JAZ impaired mutant lines, in particular changes in coronatine associated gene expression.

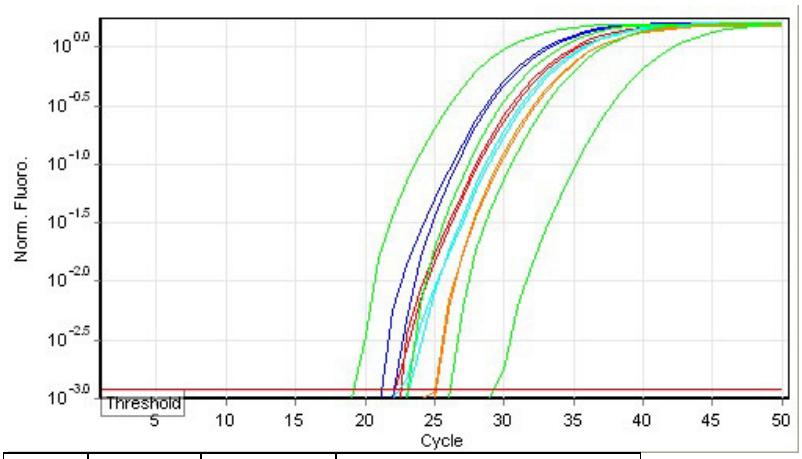
5.6: Conclusion

The *jaz* 7 mutant line has increased resistance to *P.syringae* DC3000 bacterial proliferation. Susceptibility to *P. syringae* DC3000 growth and lesion development can be restored in *jaz* 7 mutants with additional JAZ KO's indicating a role for JAZ 7 in pathogen resistance. A disparity can be observed in JAZ mutant line susceptible phenotypes and bacterial proliferation, showing only a loose correlation between them. The *jaz* 5/10 mutant line is not significantly affected by coronatine impaired *P. syringae* DC3000 compared to the wild type. Finally, that there is a relationship between coronatine associated gene expression and the *jaz* 5/10, showing an increased expression in coronatine associated genes when challenging the *jaz* 5/10 line compared to the wild type.

6: Appendix

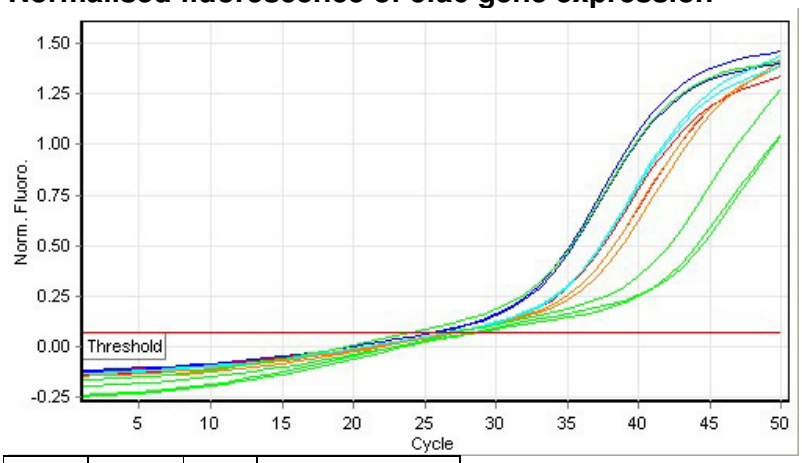
Q-PCR data

Normalised fluorescence of cmaA gene expression



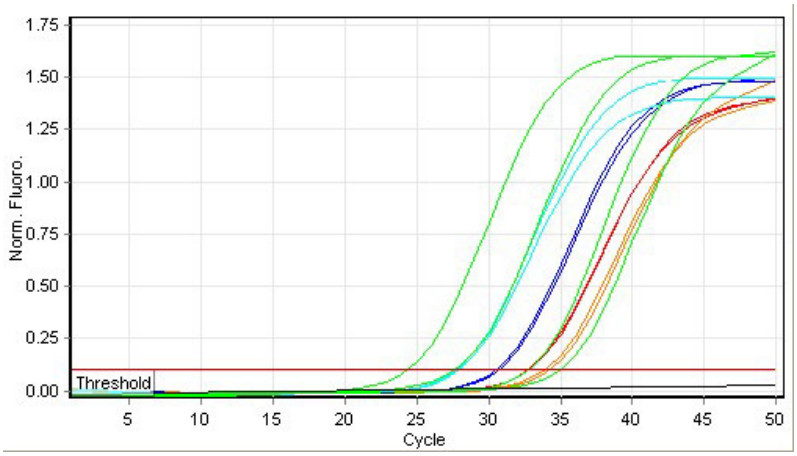
Colour	Name	Average Ct	Average Calc Conc (ng/ul)
	cmaA 2C	22.43	.001675
	cmaA 3C	25.21	0.000245
	cmaA 2D	21.83	0.002845
	cmaA 3D	23.22	0.000985

Normalised fluorescence of cfa6 gene expression



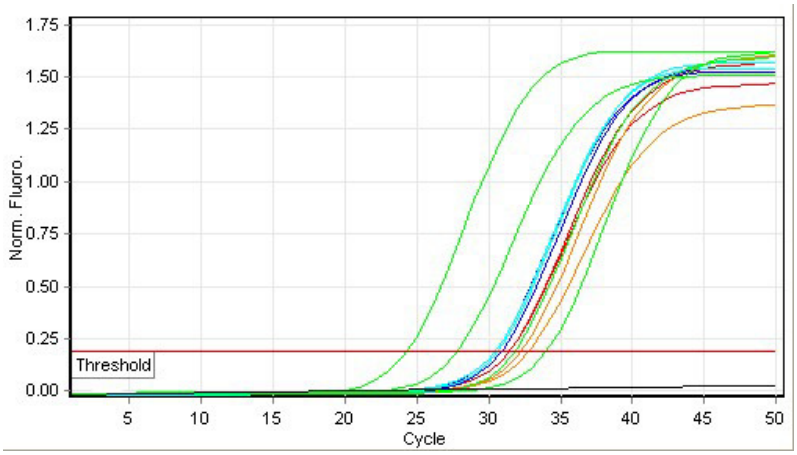
Colour	Name	Ct	Calc Conc (ng/ul)
	cfa6 2C	27.17	0.000031
	cfa6 3C	27.13	0.000033
	cfa6 2D	25.68	0.0002845
	cfa6 3D	26.82	0.000052

Normalised fluorescence of corR gene expression.



Colour	Name	Ct	Calc Conc (ng/ul)
	corR 2C	32.84	0.0000025
	corR 3C	34.11	0.000001
	corR 2D	30.66	0.0000175
	corR 3D	27.94	0.0002015

Normalised fluorescence of corS gene expression.



Colour	Name	Ct	Calc Conc (ng/ul)
	corS 2C	31.43	0.000008
	corS 3C	32.51	0.000004
	corS 2D	30.65	0.000014
	corS 3D	30.53	0.000015

Bacterial Growth data

Single JAZ mutants

Col-0

replicates	1	2	3	4	5	6
dilution	0	0	0	0	0	0
	49	10	32	75	0	0
	80	23	24	59	0	0
	90	29	34	53	0	0
	67	21	42	64	0	0
	61	40	35	62	0	0
	74	35	38	59	0	0
	7016.667	2633.333	3416.667	6200	0	0
average			4355.556	4816.667	3853.333	3211.111
stdev			2337.635	2119.792	2830.229	2980.411
SE			1349.634	1059.896	1265.717	1216.748

JAZ 1

replicates	1	2	3	4	5	6
dilution	1	1	0	0	0	0
	14	17	41	54	0	0
	15	11	46	44	0	0
	17	11	40	40	0	0
	18	9	28	40	0	0
	24	12	41	46	0	0
	12	5	52	34	0	0
	16666.67	10833.33	4133.333	4300	0	0
average			10544.44	8983.333	7186.667	5988.889
stdev			6271.659	5997.561	6566.438	6565.251
SE			3620.944	2998.781	2936.6	2680.252

JAZ 2

replicates	1	2	3	4	5	6
dilution	0	0	0	1	0	0
	30	46	30	14	0	0
	19	31	35	9	0	0
	29	33	29	12	0	0
	21	36	26	11	0	0
	29	34	40	16	0	0
	33	35	34	16	0	0
	2683.333	3583.333	3233.333	13000	0	0
average			3166.667	5625	4500	3750
stdev			453.6886	4930.602	4955.931	4798.333
SE			261.9372	2465.301	2216.36	1958.911

JAZ 3

replicates	1	2	3	4	5	6
dilution	0	0	0	0	0	0
	49	47	11	24	0	0
	52	49	11	37	0	0
	39	45	10	35	0	0
	61	44	12	38	0	0
	62	39	13	45	0	0
	28	42	12	42	0	0
	4850	4433.333	1150	3683.333	0	0
average			3477.778	3529.167	2823.333	2352.778
stdev			2026.651	1657.942	2133.678	2229.485
SE			1170.088	828.9712	954.21	910.1833

JAZ 5

replicates	1	2	3	4	5	6
dilution	1	1	0	0	0	0
	15	17	35	45	0	0
	15	21	33	60	0	0
	20	16	33	54	0	0
	11	16	28	38	0	0
	15	14	24	44	0	0
	12	18	27	45	0	0
	14666.67	17000	3000	4766.667	0	0
average			11555.56	9858.333	7886.667	6572.222
stdev			7500.617	7002.03	7497.244	7438.65
SE			4330.483	3501.015	3352.869	3036.816

JAZ 6

replicates	1	2	3	4	5	6
dilution	0	0	0	0	0	0
	23	8	31	28	0	0
	13	22	23	25	0	0
	12	25	38	17	0	0
	21	14	45	10	0	0
	31	26	30	25	0	0
	17	30	45	24	0	0
	1950	2083.333	3533.333	2150	0	0
average			2522.222	2429.167	1943.333	1619.444
stdev			878.182	740.7922	1261.646	1379.429
SE			507.0186	370.3961	564.2251	563.1495

JAZ 7

replicates	1	2	3	4	5	6
dilution	0	0	0	0	0	0
	17	5	13	10	0	0
	18	11	17	7	0	0
	22	14	16	10	0	0
	19	11	10	12	0	0
	22	16	22	4	0	0
	12	13	12	2	0	0
	1833.333	1166.667	1500	750	0	0
average			1500	1312.5	1050	875
stdev			333.3333	463.3563	711.0243	766.9384
SE			192.4501	231.6782	317.9797	313.1013

JAZ 10

replicates	1	2	3	4	5	6
dilution	1	0	1	0	0	0
	8	6	9	14	0	0
	10	9	9	17	0	0
	5	20	11	9	0	0
	5	17	8	19	0	0
	4	8	9	7	0	0
	8	16	9	22	0	0
	6666.667	1266.667	9166.667	1466.667	0	0
average			5700	4641.667	3713.333	3094.444
stdev			4037.739	3917.801	3977.548	3867.15
SE			2331.189	1958.901	1778.814	1578.758

Double JAZ mutants

Col-0

replicates	1	2	3	4	5	6
dilution	2	2	2	2	2	2
	6	5	23	7	7	3
	7	9	21	10	12	11
	10	8	17	8	5	6
	9	12	15	7	10	7
	8	4	20	6	8	4
	13	8	21	13	11	10
	88333.33	76666.67	195000	85000	88333.33	68333.33
average			120000	111250	106666.7	100277.8
stdev			65213.33	56048.51	49609.59	47051
SE			37650.93	28024.25	22186.08	19208.49

JAZ 1/3

replicates	1	2	3	4	5	6
dilution	2	2	2	2	2	2
	18	8	10	5	18	14
	21	18	16	6	8	17
	20	16	17	8	21	12
	19	14	10	9	13	11
	30	19	15	9	11	11
	18	16	9	5	18	10
	210000	151666.7	128333.3	70000	148333.3	125000
average			163333.3	140000	141666.7	138888.9
stdev			42064.76	57943.14	50318.43	45517.6
SE			24286.1	28971.57	22503.09	18582.48

JAZ 3/5

replicates	1	2	3	4	5	6
dilution	2	2	2	2	2	2
	9	6	9	5	6	8
	11	11	6	4	7	10
	12	8	6	6	7	8
	5	13	7	4	4	16
	10	12	8	6	6	10
	6	11	10	8	8	15
	88333.33	101666.7	76666.67	55000	63333.33	111666.7
average			88888.89	80416.67	77000	82777.78
stdev			12509.26	19784.72	18760.18	21951.12
SE			7222.222	9892.361	8389.809	8961.509

JAZ 2/7

replicates	1	2	3	4	5	6
dilution	1	2	2	2	2	2
	7	9	7	8	35	5
	10	11	10	7	43	6
	9	15	10	7	28	9
	14	11	13	6	39	7
	14	16	9	7	37	8
	19	11	12	4	48	9
	12166.67	121666.7	101666.7	65000	383333.3	73333.33
average			78500	75125	136766.7	126194.4
stdev			58310.23	48086.22	143988.5	131365.1
SE			33665.43	24043.11	64393.62	53629.57

JAZ 6/7

replicates	1	2	3	4	5	6
dilution	3	2	3	2	2	3
	9	15	5	14	11	9
	3	8	5	15	15	3
	5	17	6	12	19	4
	8	11	6	13	18	8
	5	6	7	15	22	4
	4	6	4	14	9	7
	566666.7	105000	550000	138333.3	156666.7	583333.3
average			407222.2	340000	303333.3	350000
stdev			261864.8	252568.3	233592.1	238157.3
SE			151187.7	126284.1	104465.6	97227.3

JAZ 5/10

replicates	1	2	3	4	5	6
dilution	2	2	2	2	2	2
	11	14	15	22	14	8
	18	15	25	20	9	9
	20	24	18	17	8	11
	21	20	22	21	16	14
	30	18	20	25	18	21
	28	18	15	21	17	24
	213333.3	181666.7	191666.7	210000	136666.7	145000
average			195555.6	199166.7	186666.7	179722.2
stdev			16187.56	15061.6	30844.59	32410.85
SE			9345.891	7530.801	13794.12	13231.67

JAZ treble mutant

Col-0

replicates	1	2	3	4	5	6
dilution	2	2	3	3	2	3
	66	49	8	7	79	9
	68	44	9	12	75	7
	70	22	10	7	61	7
	68	51	12	10	62	6
	70	44	7	9	78	3
	75	40	20	8	72	11
	695000	416666.7	1100000	883333.3	711666.7	716666.7
average			737222.2	773750	761333.3	753888.9
stdev			343617.7	289918.2	252607	226673.2
SE			198387.8	144959.1	112969.3	92538.95

Jaz 3/5/10 (T)

replicates	1	2	3	4	5	6
dilution	3	3	3	2	3	2
	13	23	20	48	48	15
	13	28	19	41	44	11
	9	31	15	37	42	14
	9	24	23	52	33	14
	18	15	29	37	40	9
	11	10	25	47	42	15
	1216667	2183333	2183333	436666.7	4150000	130000
average			1861111	1505000	2034000	1716667
stdev			558105.3	845526.4	1391183	1467145
SE			322222.2	422763.2	622156.1	598959.6

Newly isolated JAZ mutants

Col-0

replicates	1	2	3	4	5	6
dilution	2	3	3	3	3	2
	15	17	7	4	11	14
	16	6	6	5	5	15
	21	12	4	5	8	18
	22	7	6	3	4	14
	28	7	3	4	7	25
	26	3	3	2	4	19
	213333.3	866666.7	483333.3	383333.3	650000	175000
average			521111.1	486666.7	519333.3	461944.4
stdev			328300.9	276767.1	250570.5	264555
SE			189544.6	138383.5	112058.5	108004.1

JAZ 7/5

replicates	1	2	3	4	5	6
dilution	2	2	3	2	3	2
	36	15	7	13	7	12
	54	16	7	9	8	24
	49	10	5	12	10	28
	58	12	8	10	4	22
	35	21	4	12	8	34
	43	15	5	21	5	21
	458333.3	148333.3	600000	128333.3	700000	235000
average			402222.2	333750	407000	378333.3
stdev			231002.2	233084.7	259950.3	242878.6
SE			133369.2	116542.3	116253.3	99154.76

Jaz 7/10

replicates	1	2	3	4	5	6
dilution	3	3	2	2	2	2
	4	10	14	16	12	15
	3	5	19	15	14	14
	4	4	16	22	9	10
	2	6	10	23	9	19
	2	6	21	17	5	5
	5	7	20	20	11	7
	333333.3	633333.3	166666.7	188333.3	100000	116666.7
average			377777.8	330416.7	284333.3	256388.9
stdev			236486.6	215072.7	212862.8	202321
SE			136535.6	107536.3	95195.12	82597.21

JAZ 2/5

replicates	1	2	3	4	5	6
dilution	2	2	2	2	2	3
	5	12	15	11	25	6
	7	11	11	10	15	10
	7	8	18	7	20	7
	6	16	12	5	24	12
	7	14	13	12	30	15
	10	12	12	5	30	12
	70000	121666.7	135000	83333.33	240000	1033333
average			108888.9	102500	130000	280555.6
stdev			34332.25	30807.05	67030.26	373625.9
SE			19821.74	15403.52	29976.84	152532.1

JAZ 2/5/10

replicates	1	2	3	4	5	6
dilution	2	2	2	2	3	3
	9	10	9	8	12	6
	9	10	8	10	6	8
	16	18	5	12	13	8
	14	14	10	14	4	9
	13	15	9	6	5	11
	15	11	15	7	14	6
	126666.7	130000	93333.33	95000	900000	800000
average			116666.7	111250	269000	357500
stdev			20275.88	19784.72	353155.6	383103.9
SE			11706.28	9892.361	157936	156401.5

JAZ 6/5/10

replicates	1	2	3	4	5	6
dilution	2	3	3	3	3	2
	12	24	11	10	3	15
	15	22	15	11	5	13
	17	21	20	15	3	10
	7	27	14	4	4	11
	17	21	9	29	3	8
	15	15	6	10	2	17
	138333.3	2166667	1250000	1316667	333333.3	123333.3
average			1185000	1217917	1041000	888055.6
stdev			1015728	831947	821948.6	825125.2
SE			586430.7	415973.5	367586.6	336855.9

***Jaz* 5/10 mutant inoculated with *P. syringae* DC3000 and *P. syringae* DC3000 coronatine impaired mutant 2DPI**

Col-0 (DC)

replicates	1	2	3	4	5	6
dilution	3	2	3	3	3	3
	13	71	14	19	13	6
	6	96	16	11	10	10
	8	105	7	16	7	8
	8	103	14	16	10	11
	12	88	8	8	8	7
	11	66	10	10	8	14
	966666.7	881666.7	1150000	1333333	933333.3	933333.3
average			999444.4	1082917	1053000	1033056
stdev			137136.7	201017.7	186496.9	173814.8
SE			79175.93	100508.8	83403.97	70959.58

Col-0 (COR)

replicates	1	2	3	4	5	6
dilution	2	3	2	2	2	3
	12	6	40	39	53	6
	18	5	56	42	48	5
	22	9	54	41	71	4
	30	12	70	56	66	5
	18	7	40	58	66	7
	27	10	59	44	70	5
	211666.7	816666.7	531666.7	466666.7	623333.3	533333.3
average			520000	506666.7	530000	530555.6
stdev			302668.7	248562.5	221494.3	198115.2
SE			174745.8	124281.3	99055.26	80880.19

JAZ 5/10 (DC)

replicates	1	2	3	4	5	6
dilution	3	3	3	3	3	3
	13	20	13	11	7	12
	21	12	10	7	10	8
	21	16	12	16	13	10
	17	35	14	10	11	11
	19	22	14	15	10	11
	14	19	21	18	8	13
	1750000	2066667	1400000	1283333	983333.3	1083333
average			1738889	1625000	1496667	1427778
stdev			333472.2	354990.9	420548.6	412265.6
SE			192530.3	177495.4	188075	168306.7

JAZ 5/10 (COR)

replicates	1	2	3	4	5	6
dilution	2	2	2	2	2	2
	17	39	23	11	11	18
	10	53	24	20	13	23
	11	48	25	18	16	22
	27	54	33	19	12	23
	24	56	21	19	11	21
	27	51	19	25	12	20
	193333.3	501666.7	241666.7	186666.7	125000	211666.7
average			312222.2	280833.3	249666.7	243333.3
stdev			165834	149248.1	146843.6	132254
SE			95744.32	74624.06	65670.47	53992.45

***Jaz* 5/10 mutant inoculated with *P. syringae* DC3000 and *P. syringae* DC3000 coronatine impaired mutant 3DPI**

Col-0 (DC)

replicates	1	2	3	4	5	6
dilution	3	3	3	2	2	2
	11	10	6	40	39	29
	10	12	5	61	18	33
	7	8	5	57	54	25
	10	7	10	51	27	29
	8	7	4	50	44	31
	12	6	15	66	32	37
	966666.7	833333.3	750000	541666.7	356666.7	306666.7
average			850000	772916.7	689666.7	625833.3
stdev			109290.6	178130.1	241765.5	266849.4
SE			63098.98	89065.04	108120.8	108940.8

Col-0 (COR)

replicates	1	2	3	4	5	6
dilution	3	2	2	2	2	2
	9	43	26	18	13	31
	7	53	36	17	21	32
	10	28	38	18	31	16
	9	53	29	16	18	37
	8	51	44	10	18	19
	12	43	30	23	20	30
	916666.7	451666.7	338333.3	170000	201666.7	275000
average			568888.9	469166.7	415666.7	392222.2
stdev			306468.8	319989.9	301838.3	276012.6
SE			176939.9	159994.9	134986.2	112681.7

JAZ 5/10 (DC)

replicates	1	2	3	4	5	6
dilution	3	3	3	3	3	3
	13	14	11	8	10	18
	6	11	14	9	8	18
	8	10	17	5	7	14
	13	14	15	4	9	17
	11	15	20	9	9	14
	9	17	13	4	6	15
	1000000	1350000	1500000	650000	816666.7	1600000
average			1283333	1125000	1063333	1152778
stdev			256580.1	379692.9	356565.4	386927.6
SE			148136.6	189846.4	159460.9	157962.5

JAZ 5/10 (COR)

replicates	1	2	3	4	5	6
dilution	2	2	2	2	2	2
	32	28	49	19	21	24
	31	27	39	29	21	22
	26	22	58	24	23	30
	27	30	47	30	26	34
	39	23	58	27	34	27
	35	30	51	28	22	29
	316666.7	266666.7	503333.3	261666.7	245000	276666.7
average			362222.2	337083.3	318666.7	311666.7
stdev			124736.8	113581.2	106636.7	96907.75
SE			72016.8	56790.61	47689.39	39562.42

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