The role of citrate in plant-pathogen interactions.

By Tia Lynne Hendry

University of Cape Town

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Supervisor: Dr Robert Ingle

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List of Abbreviations

℃	degree Celsius
ABA	abscinsic acid
Avr	avirulent
bp	base pairs
C:N	carbon:nitrogen
cDNA	complementary deoxyribonucleic acid
cfu	colony forming untis
cm	centimetres
Col-0	*Arabidopsis thaliana* ecotype Columbia
COR	coronatine
DNA	deoxyribonucleic acid
dNTP	deoxynucleosidetriphosphate
ETI	effector triggered immunity
ETS	effector triggeres susceptibility
g	gram(s)
GABA	γ-aminobutyric acid
gDNA	genomic deoxyribonucleic acid
h	hour(s)
hpi	hours post infection
HR	hypersensitive response
IAA	indole-3-acetic acid
JA	jasmonic acid
kb	kilobase(s)
l	litre(s)
M	molar

m	metre(s)
MAPK	mitogen-activated protein kinase
min	minute(s)
mL	millilitre(s)
mM	millimolar
mm	millimetre(s)
ng	nanogram(s)
OD	optical density
PAMP	pathogen associated molecular patterns
PCD	programmed cell death
PCR	polymerase chain reaction
PR	pathogenesis-related
PRR	pattern recognition receptor
Pst DC3000	*P. syringae* pv. *tomato* DC3000
PTI	PAMP triggered immunity
R	resistance
RNA	ribonucleic acid
RNAi	interfering ribonucleic acid
ROS	reactive oxygen species
s	second(s)
SA	salicylic acid
SAR	systemic acquired resistance
T3E	type 3 effector
T3SS	type 3 secretion system
TCA	tricarboxylic acid cycle

U	units
μ	micro
μg	microgram(s)
μmol	micromole(s)
V	volts
v/v	volume per volume
w/v	weight per weight

Abstract

Bacterial plant pathogens have evolved a wide range of mechanisms to suppress the immune response that they trigger in their hosts, including the production of effectors and phytotoxins. The tri-carboxylic acid citrate, which is secreted into the apoplast by both bacterial pathogens and plant hosts has previously been shown to increase the virulence of the gram negative pathogen *Pseudomonas syringae* DC3000 (*Pst* DC3000), by acting both as a chemoattractant and as an inducer of genes associated with the type III secretion system (T3SS) and phytotoxin production. The effect of citrate on the host is less clear, though microarray analysis of *Arabidopsis thaliana* has demonstrated that application of exogenous citrate leads to the differential expression of 1876 genes suggesting that it might act as a metabolic signal for transcriptional reprogramming. In this study, functional enrichment analysis revealed statistically significant enrichment for gene ontology terms associated with defence in both citrate up-regulated and down-regulated gene sets. Furthermore this project demonstrated that exogenous citrate can increase the success of virulent *Pst* DC3000 infection in Arabidopsis; bacterial titres in plants pre-treated with citrate 24 hours prior to infection were significantly higher than those in control plants. This phenomenon was also observed in plants pre-treated with a non-metabolisable citrate analogue but not in plants pre-treated with another TCA cycle intermediate, malate, suggesting that it is citrate specific. However, it remains unclear whether the increased apoplastic citrate concentrations lead to increased bacterial titres through a suppressive effect on the host immune response, an enhanced induction of the T3SS system in *Pst* DC3000, or a combination of both. Three histidine kinases were identified as candidate citrate sensors in the Arabidopsis genome by sequence

similarity to the *Escherichia coli* CitA histidine kinase citrate sensor. However analysis of a citrate inducible marker gene in the homozygous null T-DNA knock-out lines isolated for each candidate gene revealed that none of these genes are solely required for citrate responsiveness in Arabidopsis.

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Chapter 1: Introduction

Human population levels have been rising rapidly over the last ten thousand years, and the global population is expected to plateau at around 9 billion by 2050 (Godfray *et al*., 2010). The rising population levels are placing increasing pressure on agricultural productivity in order to meet food production requirements. However food security is threatened by a number of factors, including high levels of poverty in the developing world, environmental degradation, climate change, and emergent plant pests, pathogens (Godfray *et al*., 2010) and poor farming practices. Especially damaging, plant pests and pathogens can potentially lead to a 50% decrease in major crop yields, with an estimated 10-16% of global harvest lost to plant diseases alone. When combined with postharvest spoilage and quality deterioration, these losses can become critical, particularly for resource-poor regions (Oerke, 2006). The continued practice of large scale, high density monocultures has led to a decrease in genetic variation within crop species, leaving many agriculturally important plants vulnerable to pathogen attack.

Plants come under attack from a diverse range of microbial pathogens, including fungi, bacteria and viruses, and have evolved complex immune responses to combat infection. This immune response must be overcome by these pathogens in order to successfully colonise the host. The engineering of transgenic plants capable of resisting microbial infection has the potential to reduce crop loss. It is therefore important to understand the underlying mechanisms of plant-pathogen interactions. The integration of plant immunology and pathogen infectivity is central to the manipulation of plant-pathogen interactions in order to enhance disease resistance (Dodds and Rathjen, 2010).

Pseudomonas syringae

The *Pseudomonas* genus consists of gram negative rod shaped bacteria. Species within the genus vary between soil-living and phytopathogenic lifestyles. *Pseudomonas syringae* is a bacterial plant pathogen that colonises and proliferates in the leaf apoplastic space and other aerial plant tissues by assimilating nutrients from living host cells. This bacterium can infect a wide range of plant species, including several economically important crop species, and causes a variety of symptoms, including chlorosis and necrosis of leaves and fruit, and canker of woody tissues (Rico *et al*., 2011). It has been widely used as a model organism to understand the molecular basis of (hemi) biotrophic plant disease in the model host plant, *Arabidopsis thaliana* (henceforth referred to as *Arabidopsis*). The model system between *P. syringae* and *Arabidopsis* is well studied, with both organisms possessing fully sequenced genomes and well characterised disease progression markers (Katagiri *et al*., 2002). The ability of *P. syringae* to gain entry to and proliferate inside the plant depends on its ability to synthesise toxins, hormones and effectors capable of subverting host immunity.

Plant immune response

Plants have evolved a complex immune system in order to prevent pathogen establishment and proliferation. Lacking an active circulatory system, plants rely on a multi-layered innate immune defence system which shares some features of the innate immune response in vertebrates (Jones and Dangl, 2006).

Invading pathogens are faced with pre-formed defences upon attempted entry into the host plant. These defences may be physical barriers, as with the waxy cuticles

(preventing entry into the host apoplast) and cell walls, or constitutively produced antimicrobial compounds, including antimicrobial enzymes and secondary metabolites which restrict the pathogen once inside the apoplast (Jones and Takemoto, 2004).

Once a pathogen is within its specific host, the plant relies on the recognition of non-self to induce an immune responses. The plant immune system can broadly be divided into two branches, pathogen associated molecular pattern (PAMP) triggered immunity (PTI) and effector triggered immunity (ETI), which are discussed below. A final layer of innate immunity is provided by systemic acquired resistance (SAR). Here infection of one part of a plant leads to increased resistance of the uninfected tissues to further pathogen challenge. SAR is thought to be established by the co-ordinated expression of an array of anti-microbial pathogenesis related (PR) genes (Fu and Dong 2013).

PAMP Triggered Immunity

The first branch of innate immunity (pamp triggered immunity or PTI) is activated upon the detection of conserved pathogen associated molecular patterns (PAMPS) by pattern recognition receptors (PRRs) at the plasma membrane (Chrisholm *et al.*, 2006). These PAMPS are generally regarded essential for pathogen vitality and are conserved across diverse genera, making them an effective and broad pathogen monitoring tool for plants. Several PAMPS have been intensively studied, including flg22 (a conserved epitope of the bacterial flagellin protein) (Gómez-Gómez *et al.*, 2000), chitin (a component of fungal cell walls) (Kaku *et al.*, 2006) and lipopolysaccharide (LPS) (a component of gram negative cell walls) (Zeidler *et al.*,

2004). The recognition of these PAMPS by the cognate PRRs lead to the activation of PTI (Figure 1a). This response is associated with the induction of mulitple signalling pathways, including mitogen activated protein kinase (MAPK) signalling cascades, induction of defence gene expression and stress-specific WRKY transcription factors, production of reactive oxygen species (ROS) and callose deposition (Ingle *et al.*, 2006).

These MAPK signalling cascades acts as important signalling mediators between pathogen sensors and cellular defence responses (Meng and Zhang, 2013), and functions as part of a complex signalling network that leads to the induction of PR gene expression and the biosynthesis of plant hormones including salicylic acid (SA) and jasmonic acid (JA). The synthesis of specific plant hormones is dependent on the invading pathogens trophic state. SA is active against biotrophic pathogens, which rely on living tissue to proliferate, while JA is synthesised in response to necrotrophs, which benefit from host cell death (Grant and Lamb, 2006). The specificity of these pathways is highly important, as a key component of the SA-dependent defence response is the hypersensitive response (HR) which leads to programmed cell death (PCD). It has been found that SA and JA pathways can be either antagonistic or synergistic in a dose dependent manner (Mur *et al.*, 2006), providing either negative or positive outcomes (Mur *et al.*, 2006). Activation of both pathways simultaneously result in enhanced disease resistance to *P. syringae pv. tomato* DC3000 (*Pst* DC3000) when compared to either SA or JA-dependent responses (Van Wees *et al.*, 2000; O'Donnell *et al.*, 2003). It is believed that this cross-talk between SA and JA pathways creates a flexible signalling network that enables fine tuning of defence responses while minimizing fitness costs for the plant (Mur *et al.*, 2006).

ROS produced by the PTI response assist in immunity in a number of ways. Hydrogen peroxide (H_2O_2), a common ROS, is produced in response to both biotic and abiotic stress (Doke *et al.*, 1994). It is involved in diverse defence responses, including rapid cell wall reinforcement (Bradley *et al.*, 1992), signal transduction via both calcium release into the cellular matrix (Price *et al.*, 1994), and intracellular antioxidants ascorbate and glutathione via alterations to their concentration and redox status (Foyer *et al.*, 1997), induction of defence genes (Wu *et al.*, 1997) and the HR response. H_2O_2 is also used to disrupt the redox state of cells, allowing controlled oxidation that may have an immediate antimicrobial effect (Lamb and Dixon, 1997). Incompatible plant-pathogen interactions lead to sustained H_2O_2 production which affects the cellular redox state of the host leading to reduced cell division, minimizing energy loss and risk of heritable damage (Levine *et al.*, 1994)

Effector Triggered Susceptibility

To overcome PTI, pathogens rely on the synthesis of toxins, hormones and a type 3 secretion system (T3SS) that delivers proteins (known as type 3 effectors (T3Es). These effectors of able to manipulate cell functions, including functions that are involved in the defence response that occurs after PAMP recognition (Macho and Zipfel, 2015). The proteins are injected directly from the pathogen into the host cytoplasm (McCann and Guttman, 2008) where they are termed avirulence factors (Avr) if plants possess the corresponding resistance protein (Mansfield, 2009). Suppression of PTI by effector proteins is known as effector-triggered susceptibility (ETS).

Induction of the T3SS by plant-derived metabolites

For bacteria to synthesise the T3SS and effector proteins they must first recognise they are within a potential host (i.e. switch from an epiphytic lifestyle to parasitic) (Anderson *et al.*, 2014). Early attempts to identify plant signals perceived by *Pst* DC3000 to orientate itself revealed that medium mimicking the plant apoplast was capable of inducing T3SS associated genes (Salmeron *et al.*, 1993). The medium consisted of minimal nutrient medium with an acidic pH (5.4) containing soluble sugars such as fructose (Rahme *et al.*, 1992; Tang *et al.*, 2006). Certain bioactive metabolites present in apoplast extracts (including pyroglutamic, citric, shikimic and aspartic acid) were found to strongly induce expression of the *AvrPto* effector (Anderson *et al.*, 2014) and *hrpL*, an alternative sigma factor that regulates expression of the T3SS (Tang *et al.*, 2006). These results suggested that virulent *Pst* (*Pst* DC3000) was able to perceive multiple signals derived from its host to rapidly induce expression of the T3SS.

All bioactive metabolites that induced T3SS related gene expression were organic acids but were not highly related in structure, nor were they easily converted into a common chemical intermediate (Anderson *et al.*, 2014) suggesting that *Pst* DC3000 was capable of detecting the individual metabolites. Of the metabolites associated with T3SS induction, citrate and shikimate, along with malate, were found to be strong chemo-attractants for *Pst* (Cuppels, 1988). In addition, these metabolites have been associated with additional virulence responses, including the induction of genes necessary for the biosynthesis of the phytotoxin coronatine and enhanced chemotaxis (Li *et al.*, 1998).This suggests that host-derived metabolites are capable of regulating multiple aspects of *Pst* DC3000 virulence (Anderson *et al.*, 2014). In tomato leaves the concentration of malic and citric acids were sufficient to induce

toxin synthesis (Cuppels, 1988) in *Pst* DC3000, while *Pst* DC3000 infection itself resulted in the upregulation of erythrose-4-P, a precursor of shikimate, suggesting that *Pst* is capable of both utilising existing metabolites as well as inducing the synthesis of others within their hosts. In addition to citric acid, aspartic acid is also abundant in plant tissue and both can be catabolized by *Pst* DC3000, indicating these metabolites are utilised by the pathogen in different ways at different stages in infection: as signal cues in early infections and as a nutrient source once infection is established (Anderson *et al.*, 2014). Together with contact with plant cell wall material, it is possible that the combination of soluble plant compounds and physical contact may enable the maximum induction of the *hrp* genes essential for T3SS synthesis. If this is the case, maximum T3SS induction could be induced via an interplay of a three step mechanism: environmental cues, soluble plant signals and plant cell contact (Haapalainen *et al.*, 2009).

Once the T3SS is established in the host cell's plasma membrane, T3Es are secreted into the cell cytoplasm (Figure 1b) where they are capable of manipulating cell functions, including functions involved in the PTI defence response (Macho and Zipfel, 2015). Some effectors are protein kinase inhibitors that prevent MAPK signalling and thus downstream defence activation (Xiang *et al.*, 2008); in this way effector producing pathogens are able to greatly enhance their virulence (Dodds and Rathjen, 2010). Other effectors, such as *AvrPto* from *Pst*, interact directly with the intracellular kinase domains of the PRRs, such as Flagellin sensing 2 (FLS2) and Ethylene responsive factor (ERF), themselves, preventing the autophosphorylation that occurs following PAMP recognition, and thus blocking downstream signalling (Xiang *et al.*, 2008). Effectors have also been implicated in host transcriptome reprogramming (Gohre and Robatzek, 2008).

Effector Triggered Immunity

In order to combat effector suppression of host immunity plants have evolved resistance proteins (R proteins), encoded by *R* genes, capable of detecting these effectors and are able to induce effector triggered immunity (ETI) (Abramovitch and Martin, 2004). ETI is a gene for gene response and differs from PTI in that it induces a rapid, amplified defence response, characterised by the HR and subsequent PCD. The success of ETI depends on the presence of R proteins specific to the injected effectors, which may be challenging as different strains of the same pathogen may have different effectors, and different populations of the same plant species, different R proteins.

Direct interactions function through a receptor-ligand mode of action (Dodds *et al*., 2006; Deslandes *et al*., 2003) (Figure 1c). The direct nature of the binding between the Avr and R protein results in a high genetic diversity within the effectors and host R proteins (Mackey *et al*., 2003). Direct recognition of the Avr by the R protein requires recognition of the amino acid sequence of the effector. As it is the amino acid sequence being detected, the pathogen effectors are under pressure to escape this detection, resulting in diversification of the amino acid sequence. This diversification results in a similar diversification in the R proteins as the host attempts to enable subsequent effector recognition. An example of this system is the infection of *Linum usitatissimum* (flax) by the flax rust fungus (*Melampsora lini*). Flax L567 R proteins recognise the pathogen AvrL567 effectors by amino acid sequence, and bind to them, initiating a defence response. To avoid this detection, the *AvrL567* gene has diversified into a number of different strains, with 12 identified (Dodds *et al*., 2006). This variety prevents the direct interaction of the R protein with these altered proteins. Additionally, all strains of the pathogen retain this diversified *AvrL567* gene,

indicating that the modifications either increase the fitness or do not negatively effect, of the pathogen, with the amino acid alterations affecting only the Avr-R binding, while conserving the effector structure and stability (Dodds *et al.*, 2006).

Indirect Avr-R interactions, which gave rise to the guard hypothesis (Dangl and Jones, 2006), occur through R protein recognition of Avr factor-modified host proteins (Figure 1e), where the proteins modified can be either the effectors' target or a plant decoy protein (Collier *et al.*, 2009; Chisholm *et al.*, 2006). The R proteins that recognise effectors through this method are termed guard proteins. The advantage of indirect detection of Avr factors is that the biochemical mode of action of the Avr factors is detected instead of protein sequence, preventing detection escape through Avr amino acid alterations (Mackey *et al.*, 2003). This form of recognition drastically simplifies the operation of host surveillance systems as they are able to monitor their own systems and activate defence responses once any foreign modifications to itself are detected (Jones and Dangl, 2006) These interactions are thought to be more the more common type of R protein-effector interactions, presumably as they are capable of detecting the actions of proteins rather than their exact sequence, preventing the escape of pathogens through sequence mutation .

The evolution of ETI has led to the evolution of T3Es in bacterial pathogens that are capable of suppressing both PTI and ETI (Figure 1d). In addition, pathogens have evolved multiple effectors to target specific proteins that are key players in plant defence. The effectors AvrRpm1 and AvrRpt2 both target Rpm1 interacting 4 (RIN4), a negative regulator of PTI that is guarded by two R proteins, RPM1 and RPS2. These effectors attempt to inhibit the plant immune response in different ways. AvrRpm1 modifies RIN4 through phosphorylation, presumably altering its activity (Liu

et al., 2011; Mackey *et al*., 2003). If present, the RPM1 resistance protein detects this modification and activates ETI. To prevent this activation, the second effector, AvrRpt2, which is a protease, degrades RIN4, the destruction of which destabilises the RPM1 protein preventing the activation of ETI. However, the destruction of RIN4 will activate a second R protein RPS2 if present in the host, which again triggers ETI, a nice example of the ongoing arms race between plants and their pathogens (Mackey *et al*., 2003).

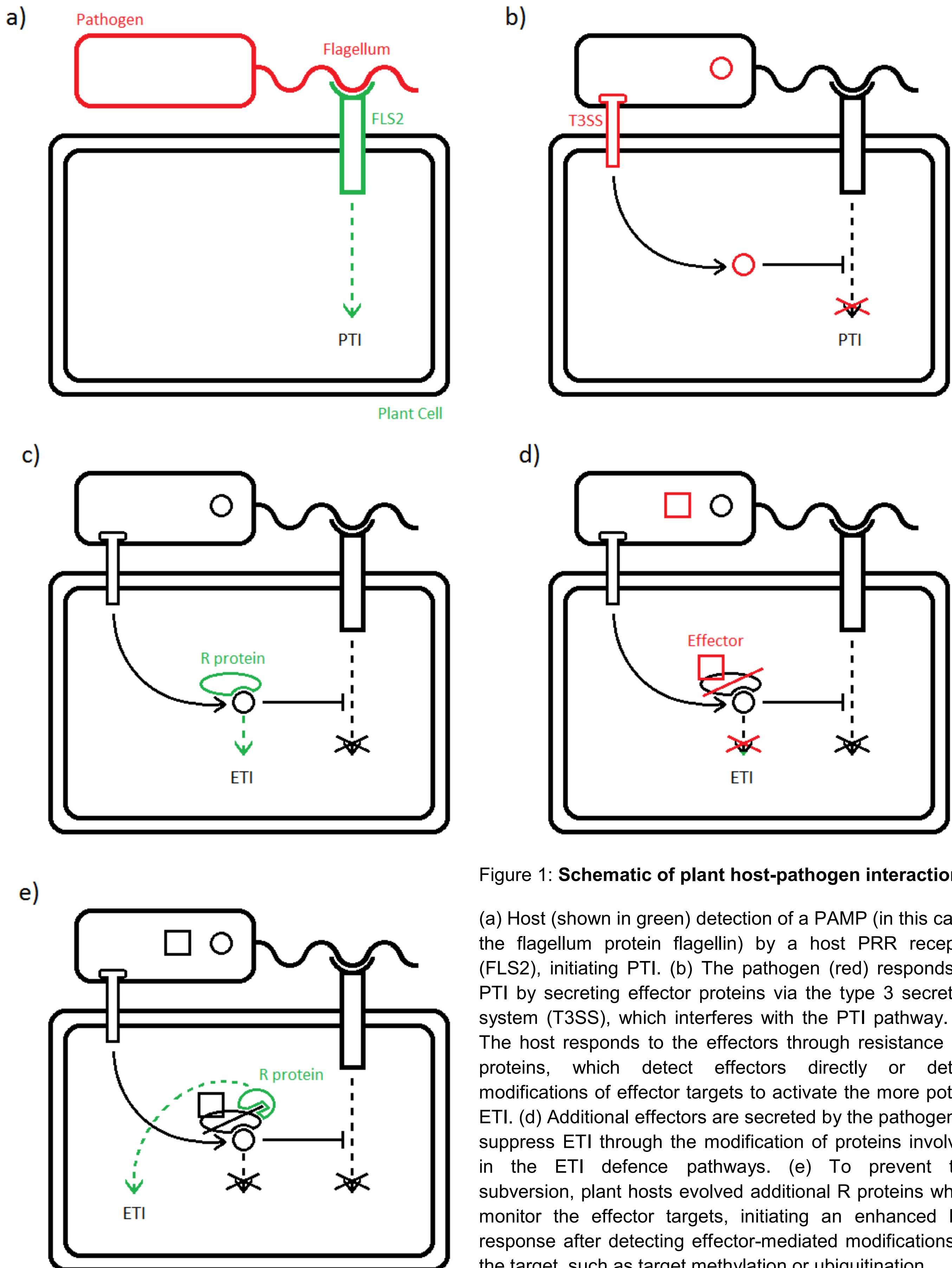

Figure 1: **Schematic of plant host-pathogen interactions.**

(a) Host (shown in green) detection of a PAMP (in this case, the flagellum protein flagellin) by a host PRR receptor (FLS2), initiating PTI. (b) The pathogen (red) responds to PTI by secreting effector proteins via the type 3 secretion system (T3SS), which interferes with the PTI pathway. (c) The host responds to the effectors through resistance (R) proteins, which detect effectors directly or detect modifications of effector targets to activate the more potent ETI. (d) Additional effectors are secreted by the pathogen to suppress ETI through the modification of proteins involved in the ETI defence pathways. (e) To prevent this subversion, plant hosts evolved additional R proteins which monitor the effector targets, initiating an enhanced ETI response after detecting effector-mediated modifications to the target, such as target methylation or ubiquitination.

Pathogen colonisation and growth in host

Subversion of stomatal closure

Numerous pathogens can survive in an epiphytic state on the plant surface, requiring entry into host tissue to initiate pathogenesis. Unlike fungi, phytopathogenic bacteria lack the ability to directly penetrate the plant epidermis, relying instead on natural openings or external wounds on the epidermis surface (Melotto *et al.*, 2006). Natural openings, such as stomata were believed to be passive ports for bacteria to gain entry into host tissue but it has been found that stomata actively close upon contact with both plant (*Pst*) and human (*E. coli*) bacterial pathogens (Melotto *et al.*, 2006). That stomatal closure isn't limited to plant pathogens in Arabidopsis suggested that the guard cells of the stomata possess the ability to detect PAMPs conserved across genera (Melotto *et al.*, 2006), including flg22 (Asai *et al.*, 2002) and LPS (Zeidler *et al.*, 2004). The ability of stomata to close as an initial response to pathogenic bacteria suggests that plants are able to sense the danger of a potential bacterial invasion and have evolved a mechanism to limit access by closing a major port of bacterial entry into the apoplast. Effective stomatal closure requires the plant hormones SA and abscinsic acid (ABA), several downstream targets, including the guard-cell-specific OST1 kinase, nitric oxide production (NO) (Figure 2), and H_2O_2 (Schroeder *et al.*, 2001; Fan *et al.*, 2004). Stomatal closure requires ABA and SA, suggesting that it is an integral part of the SA mediated defence system (Melotto *et al.*, 2006). As stomata are a major point of entry for bacteria, several plant pathogens, including *Pst* DC3000, have evolved specific virulence factors that are capable of subverting stomatal defence by forcing the stomata to reopen after the initial closure (Melotto *et al.*, 2006).

Pst DC3000 produces a variety of phytotoxins necessary for full virulence within the host (Bender *et al*., 1999) including the chlorotic phytotoxin coronatine (COR) (Ichihara *et al*., 1977), COR, an analogue of the octadecanoid precursors of JA, (Feys *et al*., 1994) induces the JA-dependent defence pathway, thereby inhibiting SA-dependent pathways (Brooks *et al*., 2005; Cui *et al*., 2005). In addition to its role in the SA-JA antagonism, COR is implicated in forced stomatal reopening through the downstream inhibition of ABA-induced stomatal closure (Melotto *et al*., 2006) utilising COI1 dependent proteolysis (Xie *et al*., 1998). (Figure 2). COR is therefore integral in the ability of *Pst* DC3000 to enter and colonize its host.

Plant hormone antagonism not limited to bacterial phytopathogens, *Botrytis cinerea*, a necrotrophic fungal pathogen, produces an exopolysaccharide (EPS) known as β-(1,3)(1,6)-D-glucan that activates the SA pathway, thus antagonising the JA defence pathway and enhancing its virulence in host plants (Mohamed *et al*., 2011).

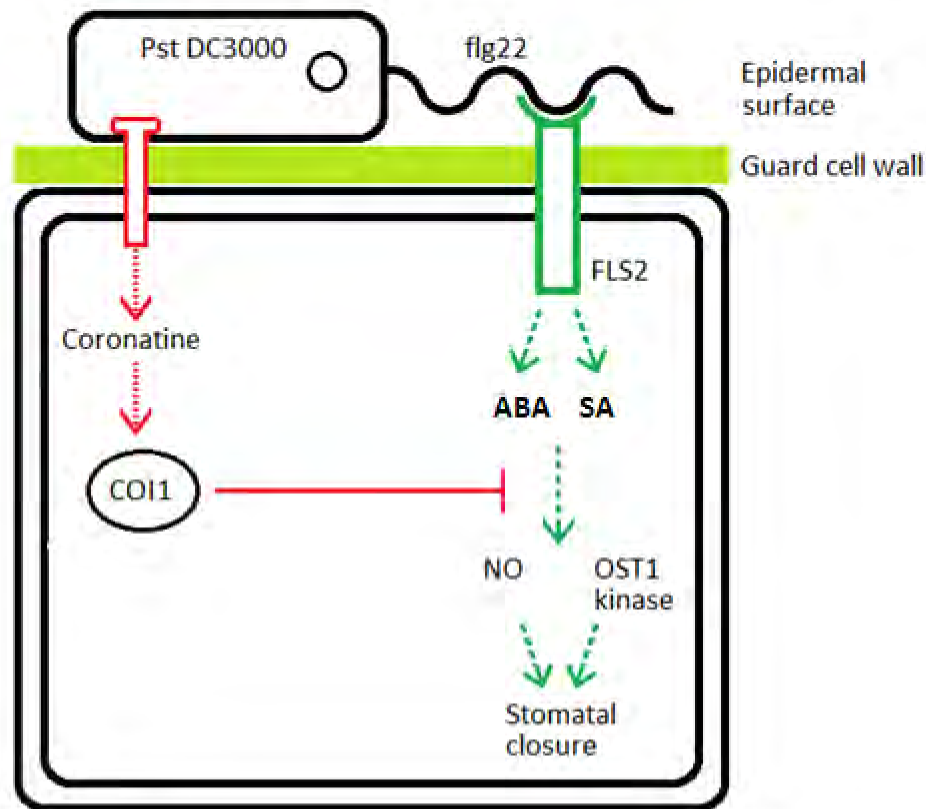

Figure 2: **Schematic showing bacterial PAMP-induced stomatal closure in Arabidopsis.**

PAMPs possessed by pathogenic bacteria, in this case flg22 from *Pst* DC3000, are recognised by PRRs spanning the guard cell wall and cell plasma membrane (the FLS2 receptor is expressed in guard cells and is shown in green) in the stomatal guard cell. PAMP recognition activates SA and ABA-regulated stomatal closure through several components (including NO and guard-cell-specific OST1 kinase).

The virulent plant pathogen *Pst* DC3000 secrets the phytotoxin COR into the guard cell via the T3SS (shown in black). The ability of COR to reopen the guard cells is dependent on COI1 the JA receptor. COR activates COI1 which functions downstream of ABA to repress stomatal closure. (Melotto *et al*., 2006)

Host nutrient manipulation

Plant biotrophic pathogens, such as *Pst* DC3000, living in the apoplast of host leaves require nutrients from the living host cells to proliferate. *Pst* DC3000 is specialised for growth within the plant but is unable to assimilate nutrients naturally present in a low concentration in the apoplast (Rico and Preston, 2008) requiring either a higher concentrations of certain nutrient in the apoplast or an enhanced uptake system. In

order to survive within the host, *Pst* DC3000 must both adapt itself metabolically as well as modify host metabolism (Block *et al*., 2008; Cunnac *et al*., 2009). Nutritional adaption of *Pst* DC3000 itself can take two forms; either through the utility and upregulation of enzymes and transport protein coding genes to facilitate assimilation of available nutrients or through the downregulation of unnecessary enzymes and transport proteins that hinder growth within the plant (Rico *et al*., 2011).

In addition to modifying its own nutrition capabilities, *Pst* DC3000 has been found to secrete effectors and toxins that adapt plant physiology and metabolism to favour its growth. These adaptations may either target and supress chloroplast function, increase nitrogen availability or modify the host cell wall. Tagetoxin impairs RNA polymerase in chloroplasts, preventing gene expression (Mathews and Durbin, 1990) while effectors such as Hop1 leads to the accumulation of heat shock proteins within the organelle, suppressing SA accumulation (Jelenska *et al*., 2010). *Pst* DC3000 also modifies the activity of glutamine and asparagine synthase in host cells, leading to an accumulation of glutamate which is converted to asparagine. This asparagine is mobilised within infected tissues, increasing nitrogen availability (Olea *et al*., 2004). The suppression of pathways leading to the deposition of lignin and diverse cell wall modifications could also facilitate the movement of water and nutrients between the cells and apoplast (Truman *et al*., 2006). Plant hormones involved in changes to plant tissues and possibly plant metabolism have also been increasingly linked to various effectors: *AvrRpt2* has been linked to indole acetic acid (IAA) signalling (Chen *et al*., 2007), *HopAM1* to ABA (Goel *et al*., 2008) and *AvrB* to JA (Cui *et al*., 2010).

Iron sequestration

While assimilation of free nitrogen and carbon may be facilitated by the use of effectors and toxins, iron accumulation poses more of a challenge. Iron is extremely insoluble in aerobic environments with a moderate pH and host concentrations of bioavailable iron (approximately 10^{-9} in mammalian cells) are far lower than those required by bacteria for optimal growth, between 10^{-6} and 10^{-7} (Guerinot, 1994). The low availability of bioavailable iron in vertebrate cells is due to the ability of free ionic iron to cause cell damage by catalysing the production of highly toxic ROS products. Free iron is therefore found bound to target metalloproteins or chelated to organic ligands, such as citrate (Brown, 1978).

In plants, iron is thought to circulate as iron (III) citrate hydroxide or iron (III) dicitrate chelates (Brown, 1978; Roschzttardtz *et al.*, 2011). In order to access this iron, bacteria are able to scavange iron host fluids using siderophores, high affinity iron carriers, that are synthesised, exported from the bacterial cell and imported once bound to iron (Miethke *et al.*, 2006), that can compete with host iron chelates. Pathogens are capable of synthesising various siderophores, but within the plant apoplast, which has a low pH (pH 5.0 – 6.0), citrate is an effective iron carrier. Assisting iron chelation via citrate is the bacterial transporter FecA, localised in the bacterial outer membrane (Marshall *et al.*, 2009). This transporter enables the high affinity import of iron (III) dicitrate into the bacterium. *Pst* DC3000 is capable of exporting mM levels of citrate in environments with iron limitation (Jones and Wildermuth, 2011).

Metabolite signalling

It has long been known that plant hormones and ionic molecules, such as Ca^{2+}, act as signalling molecules that are capable of modifying gene expression within the plant. More recently it has been found that various metabolites are also capable of acting as signal molecules.

Sugar signalling

It has been demonstrated that soluble sugars can mediate cellular responses independently of their direct metabolic role as nutrients, with both glucose and sucrose triggering gene expression changes (Jang *et al*., 1997; Moore *et al*., 2003). Elevated levels of soluble sugars upregulate genes involved in the synthesis of polysaccharides, storage proteins, respiration and defence responses (Jang and Sheen, 1994), while downregulating those involved in photosynthesis. Plant damage caused by pathogen infection (Chou *et al*., 2000) or wounding was correlated with increases in soluble sugar, particularly hexose, at the damage site. This increase in sugar concentrations induces the transcriptional activation of Pathogenesis Related (PR) protein-coding genes in Arabidopsis (Thibaud *et al*., 2004). It has been proposed that carbohydrate levels and pathogen response may be interrelated, suggesting that defence genes are activated in response to detection of metabolic modifications and changes in carbon flux (Roitsch, 1999; Schneider, 2002). Another defence related role soluble sugar may play lies in the final component of SA-dependent defence responses: the HR. Hexokinase, an enzyme that catalyses the initial step in glucose metabolism, has been identified as a direct metabolite sensor of hexose and glucose concentrations that activates sugar-related gene expression

(Moore *et al*., 2003). Hexokinases localise to different organelles within the plant (Galina *et al*., 1995), including the mitochondria. Mitochondria play a key role in the induction of the HR response in plants, with mitochondria-associated hexokinases in tobacco found to regulate its induction (Kim *et al*., 2006), with interruption of hexokinase function activating PCD in plant cells. This hexokinase-mediated cell death pathway involves ROS, cytochrome c release from mitochondria, activation of caspase-like activities, and transcriptional induction of cell death–related genes (Kim *et al*., 2006).

GABA and metabolite signalling

The identification of molecules functioning as metabolite signals in plants is still relatively new. One way to identify possible metabolites is to look at conserved metabolites between kingdoms, which have been found to function in signalling pathways. γ-Aminobutyric acid (GABA), a 4-carbon non-protein amino acid conserved from bacteria to plants and vertebrates and involved in nitrogen metabolism, had been discovered in plants over half a century ago (Steward *et al*., 1949), but interest shifted to animals after it was discovered to play a major role in neurotransmission (Bouche and Fromm, 2004). GABA is metabolised via the GABA shunt, a short pathway composed of three enzymes bypassing two steps of the TCA cycle, in both plants and animals (Bouche and Fromm, 2004). More recently, GABA concentration was seen to rapidly increase in response to biotic and abiotic stress in plants (Shelp *et al*., 1999), including pathogen infection. This abundance of GABA and glutamate leads to changes in nitrogen metabolism in the host (Ward *et al*., 2010), which suggests GABA may have a significant impact on metabolic control.

Possible roles of this metabolite include involvement in C:N balance due to its role in general nitrogen metabolism, regulation of cytosolic pH, as GABA accumulates in response to cytosolic acidification, and biotic defence, with GABA levels increasing in response to physical stimulation or damage (Janzen *et al.*, 2001). The accumulation of GABA in transaminase deficient mutants, which are unable to metabolise GABA, led to an increased resistance to *Pst* DC3000 (Park *et al.*, 2010). This resistance was due to the high concentrations of GABA within the mutants suppressing T3SS expression, suggesting that *Pst* DC3000 requires a rapid turnover of GABA for growth and virulence (Vanacker *et al.*, 1998).

Tricarboxylic acid cycle intermediates as metabolite signals

Tricarboxylic acid (TCA) intermediates citrate, succinate and fumerate have been found to have signalling functions in humans (Hewitson *et al.*, 2007), with iron citrate competitively inhibiting tyrosine (Tyr) phosphatases and enhancing MAPK signalling (Gomez *et al.*, 2010). In yeast, TCA intermediates are also seen to have an important function in metabolite signalling, as well as conserved functions as TCA intermediates (McCammon *et al.*, 2003). More than 400 genes were found to be highly responsive to TCA cycle defects in mutant yeast, suggesting that nuclear gene signalling is responsive to TCA cycle function. .

TCA intermediates reflect both the metabolic and redox status of the cell and are transported between cellular compartments within the cell, and are therefore good candidates as signalling molecules in plants (Fernie *et al.*, 2004; Meyer *et al.*, 2010).In a study comparing changes in the transcriptome in Arabidopsis induced by exogenous application of TCA metabolites, citrate (and to a much lesser extent

malate, with 327 altered transcripts), led to differential expression of 1,876 genes (Finkemeier *et al*., 2013). The transcriptional responses differed between the two metabolites, with several genes displaying inversely regulated transcripts between the treatments. This is not unexpected as while both are TCA intermediates, they possess different chemical properties and have partially different cellular functions (Finkemeier *et al*., 2013). The strongest upregulated transcripts included an unknown protein AT1g73120, hence referred to as *citrate-induced 1* (*CI1*), and *ferritin 1* (*FER1*), involved in iron homeostasis, which were upregulated in response to treatment with exogenous citrate. Interestingly, these transcripts did not display the same change after treatment with isocitrate or any other organic acid or sugar, and were instead down-regulated. This indicates that the transcript response of these two genes is specific to citrate, and that the plant can distinguish between citrate and isocitrate in the cell (Finkemeier *et al*., 2013). The nonmetabolizable citrate analogue tricarballylate (Wolffram *et al*., 1993), which can be transported into cells and organelles via citrate transporters, induced a similar transcript profile to citrate, including the upregulation of the citrate-specific *FER1* and *CI1*. This indicates that the observed changes to the transcriptome are caused by citrate itself, and not a breakdown product. In addition, plants expressing a dexamethasone (dex) inducible mitochondrial NADPH-isocitrate dehydrogenase RNA interference construct displayed a significant increase in citrate content within several hours of dex treatment but this did not result in a strong transcriptional response – only 105 of the 1876 genes (5.6%) responsive to extracellular citrate showed a significant change in expression in the RNAi lines (Lee Sweetlove, personal communication). These data strongly suggest that plants have evolved a mechanism to detect extracellular rather than intracellular citrate.

Interestingly, the transcriptome changes observed after citrate infiltration were in the same direction as those seen after treatment with avirulent *Pst* DC3000 (Finkemeier *et al.*, 2013). Cluster analysis revealed that the highest similarity, in five of ten clusters, was with 24 hour treated *Pst* DC3000 infected leaf samples (Wang *et al.*, 2008), and leaf samples infected with flg22. This similarity may have arisen due to downregulation of several transcripts related to pathogen defence, such as the PR5 transcript (Finkemeier *et al.*, 2013), as well as for transcripts enriched for photosynthesis.

While the transcriptional response to citrate might occur in response to plant-produced citrate exported into the apoplast, an alternative source of apoplastic citrate is secretion from plant pathogen bacteria. It is known that *Pst* secretes citrate into the host apoplast to act as a high affinity iron chelator. However, its function solely in the role of iron scavenging is questionable, as triple mutants lacking iron scavenging siderophores, including citrate, grow normally in tomato plants and are able to cause same severity of disease symptoms as wild-type strains (Jones and Wildermuth, 2011). Given that the transcriptome profile of plants treated with exogenous citrate show similarities to those infected with *Pst*, suggest citrate may have an alternative role, perhaps in the suppression of host defences.

Project objectives

The aim of this project was two-fold: firstly, to use the Arabidopsis-*Pst* pathosystem to determine whether pre-treating plants with exogenous citrate leads to altered susceptibility to *Pst* DC3000 and secondly, given the strong transcriptional response that occurs in Arabidopsis to citrate to investigate possible receptors for apoplastic citrate in Arabidopsis.

Chapter 2: Materials and Methods

Plant Material and Growth Conditions

Plant lines

The *Arabidopsis thaliana* Columbia (Col-0) ecotype was used as wild type in this project. Transfer DNA (T-DNA) insertion lines (Table 1) were obtained from the Nottingham Arabidopsis Stock Center (NASC) (Scholl *et al.*, 2000).

Table 1: **T-DNA insert plant lines used for the duration of this project**.

Information on the T-DNA insert lines include the interrupted gene name, locus of the interrupted gene, T-DNA line name and the name used in this project.

Gene name	Locus	T-DNA line	Name
HK1	*At2g17820*	Salk_000976	*hk1-1*
		Salk_000977	*hk1-2*
HK2	*At5g35750*	Salk_052531C	*hk2-1*
		Sail_1289_G06	*hk2-2*
HK5	*At5g10720*	Salk_076188C	*hk5-1*
		Sail_50_H11	*hk5-2*
CS2	*At3g58750*	Salk_074880	*cs2-1*
		Salk_078815	*cs2-2*

Soil-grown plants

Seeds were sown on a 1:1 potting soil mixture of peat (Jiffy Products International Stage, Norway) and vermiculite and covered with cling film to prevent soil desiccation. The plant trays were stratified in the dark at 4°C for 48 h and then moved to a growth chamber with the following conditions: constant 22°C temperature, 16:8 h light:dark cycle, 80-100 μmol photon.m^{-2}.sec^{-2} of light and at 55% relative humidity (hereafter termed standard growth conditions). Cling film was removed after 7 days and excess plants removed, to leave one seedling per pot. Experiments were performed on plants 4 weeks after germination.

Agar-plate grown plants

Seeds to be plated on plant agar plates were placed in 1.5 mL microfuge tubes and washed with 70% (v/v) ethanol for 5 min, with periodic inversion. After washing, the 70% ethanol was discarded and replaced with 100% ethanol which was immediately aspirated off. Seeds were allowed to dry in the microfuge tubes in a sterile laminar flow unit, followed by suspension in 0.1% (w/v) sterile bacteriological agar before being sown onto half strength Murashige and Skoog (MS) agar plates (0.8% w/v agar). MS pH was adjusted to 5.5 using 0.1 M potassium hydroxide (KOH) prior to the addition of agar. Plates were stratified for 48 h at 4°C in the dark, after which plates were placed at standard growth conditions.

Kanamycin selection

Salk T-DNA inserts contain a kanamycin selection marker (*NptII*). Plants expressing the *NptII* gene are resistant to the antibiotic kanamycin, and *NptII* homozygous lines (which display 100% resistance) can thus be identified by sowing on MS agar (0.8% w/v) containing 50mg/mL kanamycin. Kanamycin sensitive plants fail to germinate or display reduced leaf and root growth between 1 and 2 weeks of growth.

BASTA selection

Plants expressing the *Bar* gene (which is present in the Sail T-DNA insert) are resistant to herbicides (such as Basta) containing glufosinate ammonium (dl-phosphinothricin), which can be used to select for Sail T-DNA insert containing plants (again homozygous lines will display 100% resistance). Seven-day-old *A. thaliana* plants were sprayed with a 0.03% (w/v) Basta solution until damp. Two more rounds of herbicide spraying took place 48 and 72 h after the first application respectively, after which resistant plants could be distinguished by healthy green leaves, and sensitive plants by chlorosis.

Bacterial Strains and Plant Infections

Pseudomonas syringae

Two *Pseudomonas syringae* pv *tomato* strains were used for the duration of this project, the virulent strain *Pst* DC3000, and the type three secretion system mutant *Pst* DC3000 *hrpA*. Both strains were cultured in King's Broth (KB) medium (King *et al*, 1954) on either plates (1.2 % w/v agar) or in liquid media, containing 50 µg/mL rifampicin (*Pst* DC3000) (Whalen *et al.*, 1991) or 20 µg/mL rifampicin and 50 µg/mL kanamycin (*Pst* DC3000 *hrpA*). Bacteria were incubated at 30°C for 15 h overnight, with constant shaking for liquid cultures.

TCA cycle metabolite infiltration

Three leaves from four-week-old Col-0 plants were pressure infiltrated (with a needleless syringe) on the abaxial leaf surface with either 1 mM MES (pH 5.5), 10 mM citrate (10 mM citric acid, 1 mM MES, pH 5.5), 10mM malate (10 mM malic acid, 1 mM MES, pH 5.5) or 10 mM KCl and returned to standard growth conditions for 24 h prior to infection with *Pst*.

A. thaliana infection assays

Leaves of four-week-old soil grown *A. thaliana* plants were pressure inoculated with either *Pst* DC3000 or *Pst* DC3000 hrpA strains, according to the following protocol. A 5 mL overnight *Pst* culture was centrifuged to pellet cells which were then washed and resuspended in 10 mM $MgCl_2$. The bacterial cell suspension was diluted to give a final OD_{600} of 0.002 corresponding to10^6 colony forming units (cfu)/mL (Katagiri *et*

al, 2002), in a total volume of 50 mL. The same leaves pre-infiltrated 24 h previously with the selected metabolite (as described above) were pressure inoculated with the bacterial suspension using a needleless syringe on the abaxial leaf surface. Five plants per metabolite treatment per time point (for harvesting at either 4 or 48 h post infection, hpi) were inoculated and an additional plant per treatment was infiltrated with 10 mM $MgCl_2$ as a mock infection negative control. Inoculated plants were covered with cling film and placed back under standard growth conditions for 48 h. Infiltrated leaves were harvested at 4 h and 48 hpi to analyse bacterial growth. Bacterial titres were determined by grinding three 0.2 cm^2 leaf discs per plant (each from a single inoculated leaf) in 1 mL 10 mM $MgCl_2$ and making a serial dilution of the resulting bacterial suspension (down to 1 x 10^{-6} for *Pst* DC3000, and 1 x 10^{-3} for *Pst* DC3000 *hrpA*). Each dilution was then plated onto KB agar plates (50 µg/mL rifampicin (*Pst* DC3000) or 20ug/mL rifampicin and 50ug/mL kanamycin (*Pst* DC3000 *hrpA*) and incubated for 48 h at 30°C. Colony forming units were recorded for each sample and bacterial titres (cfu per cm^2 leaf area) calculated.

Nucleic acid analysis and manipulation

Isolation of genomic DNA from *A. thaliana*

A. thaliana genomic DNA (gDNA) was isolated using the Edwards extraction protocol (Edwards *et al.*, 1991). The following modifications were made to the original protocol: leaf tissue was harvested as 4.5 mm diameter discs and homogenised directly in 500 µL Edwards's extraction buffer.

DNA amplification by polymerase chain reaction

For the genotyping of T-DNA insertion lines, gDNA was amplified using Supertherm DNA Polymerase (Separations Scientific SA Pty Ltd., Honeydew, South Africa) in standard polymerase chain reactions (PCR). Table 2 lists the primers used to amplify genomic DNA loci, describing the genetic locus, and primer sequence. Each PCR reaction was made up to a total volume of 20 µL and contained 1.5 mM $MgCl_2$, 0.4 µM of each primer, 0.2 mM dNTPs, 1 x PCR Buffer, 0.5U Supertherm DNA Taq polymerase and 100 to 200 ng template DNA. PCR reactions were performed according to the following cycling protocol: initial denaturation at 94°C for 5 min, followed by 35 cycles (of 94°C for 15 s, 55℃ for 30 s, and 72°C for 15 s per kb of target region) with a final elongation at 72°C for 10 min. All reactions were carried out in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, USA).

Table 2: **PCR Primers used for genotyping of T-DNA insertion lines.**

Each PCR primer pair is shown with the locus of its gene to be amplified and primer sequence in the 5' to 3' direction.

Primer Name	**Locus**	**Primer Sequence**
Hk1-LP	*At5g10720*	TTCACAAGACTCACACATGGG
Hk1-RP		ACATCTCCACGCAAAATCATC
Hk2-1-LP	*AT5G35750*	CTCAAGGTTTTCAGGTTGCTG
Hk2-1-RP		TGGGTAATGAGAGACACCAGC
Hk2-2-LP		TGGATAAAAAGTTTCAGGCCC
Hk2-2-RP		GACATAGTCCCAAACGCAAAG
Hk5-1-LP	*AT2G17820*	ATGTTGAATCGGTTGCAAAAG
Hk5-1-RP		AGGAAAAAGCCGAGACAGAAG
Hk5-2-LP		TACTCTGCTGGATTCGAATGG
Hk5-2-RP		AGAGCGAGATGCTACAGCTTG
Cs2-LP	*AT3G58750*	TGCAAAAGCAAAGGATGGTAC
Cs2-RP		TCCTTTGAGTGATCCATGAGG
Salk LBb1.3		ATTTTGCCGATTTCGGAAC
Sail LB3		TAGCATCTGAATTTCATAACCAATCTCGATACAC

DNA electrophoresis

DNA electrophoresis was performed using a 1% (w/v) agarose gel prepared using 1 x TAE buffer (40 mM Tris, 1 mM EDTA, 0.11% (v/v) glacial acetic acid) containing 0.016 µg/mL ethidium bromide (EtBr). DNA was separated alongside either a 1 Kb or 100 bp size marker (O' Gene Ruler ™ DNA ladder, Fermentas, Ontario, Canada) in

1 x TAE buffer at 100 V, after which it was visualised with a long wavelength (365 nm) Gel Doc™ XR UV transilluminator (Bio Rad Laboratories, UK).

Purification of PCR products

PCR samples were purified with a Wizard® DNA Clean-Up System kit according to manufacturer's guidelines (Promega, USA).

DNA sequencing and analysis

All DNA sequencing was performed at the Central Analytical Facility (Stellenbosch, South Africa) on an ABI3730xl DNA analyser (Applied Biosystems, Foster City, USA). The sequence data was analysed using Chromas software (Version 2.01, Technelysium Pty Ltd, Queensland, Australia) for multiple sequence alignment against sequences obtained from The Arabidopsis Information Resource (TAIR) database (http://arabidopsis.org) or the National Centre of Biotechnology (NCBI) database (http://blast.ncbi.nlm.nih.gov). Information on the predicted T-DNA insertion site for each line was obtained from the SALK T-DNA database (http://signal.salk.edu/cgi-bin/tdnaexpress).

RNA extraction

Total RNA was isolated using TRIzol reagent (100 mM NaAc pH 5.2, 800 mM guanidine thiocyanate, 400 mM ammonium thiocyanate, 5% glycerol (v/v) and 38% phenol (v/v) pH4,) according to the TRIzol reagent protocol (Invitrogen, Carlsbad, USA) with the following modification: samples were homogenised using 3 stainless

steel ball bearings in 1 mL TRIzol reagent by subjection to 4 min of mechanical disruption in a paint shaker.

RNA electrophoresis

To assess RNA integrity, the RNA was separated using a formaldehyde-agarose denaturing gel containing 1 x MOPS (0.4 M 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.1 M NaAc, 10mM EDTA, pH 7), 1.2% (w/v) agarose and 6% (v/v) 37% formaldehyde. The RNA samples were mixed with 0.2 volumes of RNA sample application buffer (4 x MOPS, 2.7% (v/v) 37% formaldehyde, 30.8% (v/v) formamide, 0.01 mg/mL EtBr and trace bromophenol blue) denatured at 65°C for 5 min and snap cooled on ice prior to gel electrophoresis. Separated RNA was visualised using a Gel Doc™ XR UV transilluminator (Bio Rad Laboratories, UK).

DNase treatment of RNA

RNA was treated with DNAse from the Turbo DNA-free™ kit (Life Technologies, California, USA) according to the manufacturers guidelines with the following modifications: 2.5μg of total RNA and a half volume of Turbo DNase enzyme were used.

cDNA synthesis

cDNA synthesis was performed using Superscript III Reverse Transcriptase (Life Technologies, California, USA) according to the manufacturer's protocol with the following modifications: 1 μg total RNA and a half volume of the Superscript™ III enzyme were used in a final reaction volume of 20 μl.

Analysis of gene expression by RT-PCR

In order to confirm that homozygous T-DNA insertion lines were true knock-outs of the gene of interest end point RT-PCR was used. One µL of cDNA generated from RNA extracted from the putative knock-outs was used as template in a standard PCR reaction. Similarly to examine *CI1* expression in the knock-out lines, one µL of cDNA generated from plants treated with either MES (1 mM MES, pH 5.5) or citrate (1 mM MES, 10 mM citrate, pH 5.5) was used as template in a standard PCR reaction.. Table 3 lists the primers used to amplify cDNA, describing the genetic locus, primer sequence and annealing temperature.

Table 3: **cDNA specific primers used for the duration of this project.**

Each PCR primer pair is shown with the locus of its gene to be amplified, primer sequence in the 5' to 3' direction and primer annealing temperature

Primer name	Locus	Primer Sequence	Annealing Temp (°C)
Hk1-KO F	*AT5G10720*	CGGAGGAACTGGACTTGGAC	62
Hk1-KO R		GGCATCTGGCAGTCCATGAG	
Hk2-KO F	*AT5G35750*	ATGGATGGATTTGAAGCGACA	59
Hk2-KO R		TCTCACTCAACCAGACGAGG	
Hk5-KO F	*AT2G17820*	CCAGTTCTGGTCATTGCCCT	62
Hk5-KO R		TGTTGCAAACACTCTCTCAGTTTTTGC	
Cs2-KO F	*AT3G58750*	TGAATAATCTTATCACCGTCGAG	51
Cs2-KO R		CATTAACAGGAATAAAAGCGCAA	
Act2 big F	*AT3G18780*	CACCATGTTCGTCAAGCCGAT	54
Act2 big R		ATAGAGACTTCGAGTCTTCGACGAG	
CI1 big F	*AT1G73120*	CAGTGGTCGTACAACCGGTATTG	54
CI1 big R		AGAGTTTGTCACACACAAGTGCATC	

Statistical analysis

Bacterial titres were analysed using StatSoft Statistica software (version 11) with natural log transformation of the data prior to analysis of variance (ANOVA). Fisher LSD post hoc tests were conducted to allow identification of significantly different mean values within an experiment. One-sample t-tests were used to compare mean relative bacterial titres from Malate and Citrate treated plants to MES control (set as 1).

Chapter 3: Results

<u>**Functional enrichment analysis of genes differentially expressed in response to extracellular citrate reveals over-representation of GO terms associated with defence**</u>

It has been shown that plants are able to perceive citrate (Finkemeier *et al*., 2013), but that they respond strongly to changes in extracellular citrate levels and only modestly to changes in intracellular levels. One potential source of extracellular citrate is from secretion of this metabolite into the apoplast by plant pathogenic bacteria including *P. syringae* (*Pst*) DC3000 at millimolar concentrations (Jones and Wildermuth, 2011). This secreted citrate was thought to play a role in iron scavenging from the apoplast, however mutants lacking the Fe-cit uptake system show normal growth and pathogenicity (Jones and Wildermuth, 2011) suggesting that it may play another role. The hypothesis proposed in this project is that exogenous citrate may serve to repress the host defence response against plant pathogens.

In order to investigate this, the 1876 genes identified as citrate-responsive in Arabidopsis by Finkemeier *et al*., (2013) were analysed for any evidence of enrichment for gene ontology (GO) terms associated with the host defence response. Of the 1876 genes differentially expressed in response to citrate, 1043 were upregulated, with the remaining 833 downregulated (Finkemeier *et al*. 2013). Functional enrichment analysis of both sets of genes identified several defence linked GO terms that are over-represented in the DEG list (Table 4).

Table 4: **Defence related GO term enrichment in genes differentially expressed in response to citrate.**

Each term is represented by its GO numerical code and whether it was up or down-regulated upon treatment with citrate. The genes changed % is the percentage of differentially expressed genes (DEG) that are annotated with this GO term, while the total percentage of genes annotated in the rest of the genome with this term is indicated by Genome %. The adjusted p value is from a post-hoc Fisher's exact test looking for evidence of over-representation.

Term	GO numerical code	Regulation	Genes changed %	Genome %	Adjusted P value
Defence responses	GO:0006952	Up	5.08	2.58	1,28E-04
		Down	5.40	2.58	6.76024E-4
Response to other organism	GO:0051707	Up	4.99	1.46	2,67E-11
		Down	3.12	1.58	4.60611E-2
Response to Bacterium	GO:0009617	Up	2.68	0.70	4,98E-07
Response to Fungi	GO:0009620	Up	1.44	0.36	3,29E-04

Defence genes that were differentially expressed include *FLG22-induced Receptor like Kinase* (*FRK1*), *WRKY11*, *Accelerated Cell Death 6* (*ACD6*), *Bri1-Associated receptor Kinase* (*BAK1*), *Botrytis-Induced Kinase 1* (BIK1*)* (Finkemeier *et al.,* 2013) (Table S1, Supplementary Data), indicating that genes involved in multiple processes in the plant defence response were affected by citrate. *FRK1* contains a WRKY transcription factor and LRR receptor kinase and is known to be induced by pathogens, pathogen derived elicitors or salicylic acid (Asai *et al*., 2002) while *WRKY11* is a known negative regulator of the basal plant immune system (Journot-Catalino *et al*., 2006). The genes *ACD6* and *BAK1* are both known to be involved in cell death, with *ACD6* involved in SA mediated cell death (Rate *et al*., 1999) and

BAK1 involved in regulation of microbial infection induced cell death containment (Kemmerling *et al.,* 2007). Also involved in the defence response, BIK1 links defence response regulation to growth and development through the signalling of cellular factors required for defence against pathogen infection (Veronese *et al*., 2006).

Although the potential effect of citrate on the host defence response is unclear as the GO defence terms are enriched in both up and down-regulated gene sets, it is clear that citrate can impact the expression of defence genes in Arabidopsis. As *Pst* DC3000 is known to secrete citrate into the apoplast (Jones *et al*., 2011), it was decided to test whether pre-treatment of Arabidopsis with citrate influences susceptibility of this plant species to pathogen attack.

Pre-treatment of Arabidopsis with citrate leads to increased susceptibility to *P. syringae.*

In order to investigate whether pre-treatment with extracellular citrate can influence the outcome of plant-pathogen interactions, whether negatively or positively, the *P. syringae* DC3000-Arabidopsis pathosystem (Katagiri *et al*. (2002) was employed. Leaves of four week old *A,thaliana* Col-0 plants were pressure infiltrated with either citrate (10 mM citrate in 1 mM MES, pH 5.5) or MES (1 mM, pH 5.5) 24 h prior to inoculation with *Pst* DC3000. Plants pre-treated with citrate displayed significantly higher bacterial titres at 48 hpi (hours post infection) (p=0.03) than those treated with the control buffer MES (Figure 3). These findings suggest that apoplastic citrate can modulate the outcome of this plant-pathogen interaction.

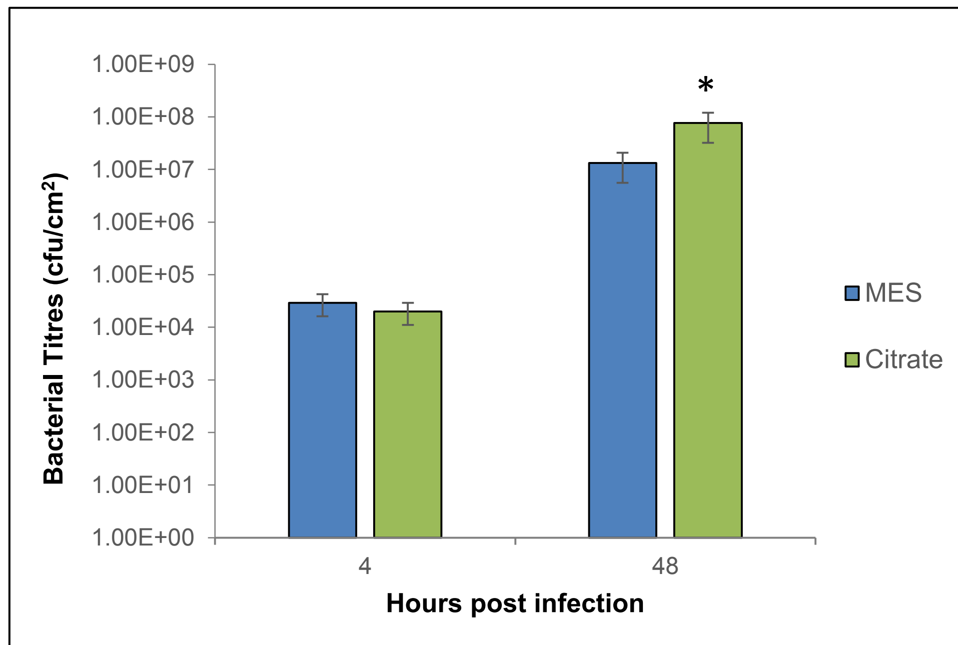

Figure 3: **Citrate pre-treatment results in increased *Pst* DC3000 bacterial titres.**

Leaves of 4 week old *A. thaliana* plants were pressure infiltrated with either citrate or MES 24 hours before infection with *Pst* DC3000. Bacterial titres were determined at 4 and 48 hpi. Bars represent the standard error of the mean (n=4). The star represents a significant difference between bacterial titres at 48 hpi ($p<0.05$) as determined by Student's t-test.

While citrate pre-treatment does appear to result in increased *Pst* DC3000 titres, there were several limitations of the initial experiment. For example, the solutions used differ in osmotic potential, and it is also possible that the increased *Pst* titre could result simply from the provision of a C source rather than through any effect on host defence responses. To exclude the first possibility the effect of pre-treatment with 10 mM KCl in 1 mM MES pH 5.5 was tested, and was found to have no effect on Pst DC3000 titres (Figure 2).

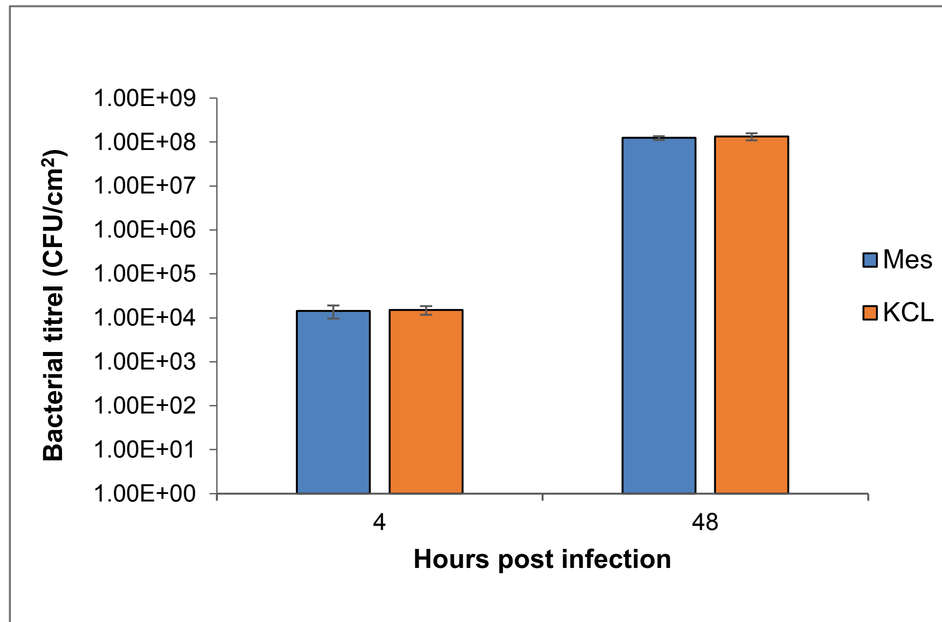

Figure 4: **KCl pre-treatment shows no change in bacterial titres.**

Leaves of 4 week old *A. thaliana* plants were pressure infiltrated with either MES or KCL 24 hours before infection with *Pst* DC3000. Bacterial titres were determined at 4 and 48 hpi. Bars represent the standard error of the mean (n=4).

To test the second hypothesis, another TCA metabolite, malate, was assayed for the ability to influence *Pst* DC3000 titres in Arabidopsis. While exogenous citrate caused major re-progamming of the Arabidopsis transcriptome (1876 genes differentially expressed), exogenous malate was found to have only a minor effect (Finkemeier *et al.,* 2013). As such, malate was chosen as an appropriate a control to test whether the response to citrate observed in Figure 1 is specific to this TCA metabolite. Plants were treated as described above, with the addition of malate (10 mM malate in 1 mM MES, pH 5.5) as a second metabolite pre-treatment. While bacterial titres appeared higher in the citrate pre-treated plants in each independent assay (Figure 3A and B), statistical analyses (Student's t-test) of the titres revealed no significant difference between citrate-treated and control plants (p=0.21).

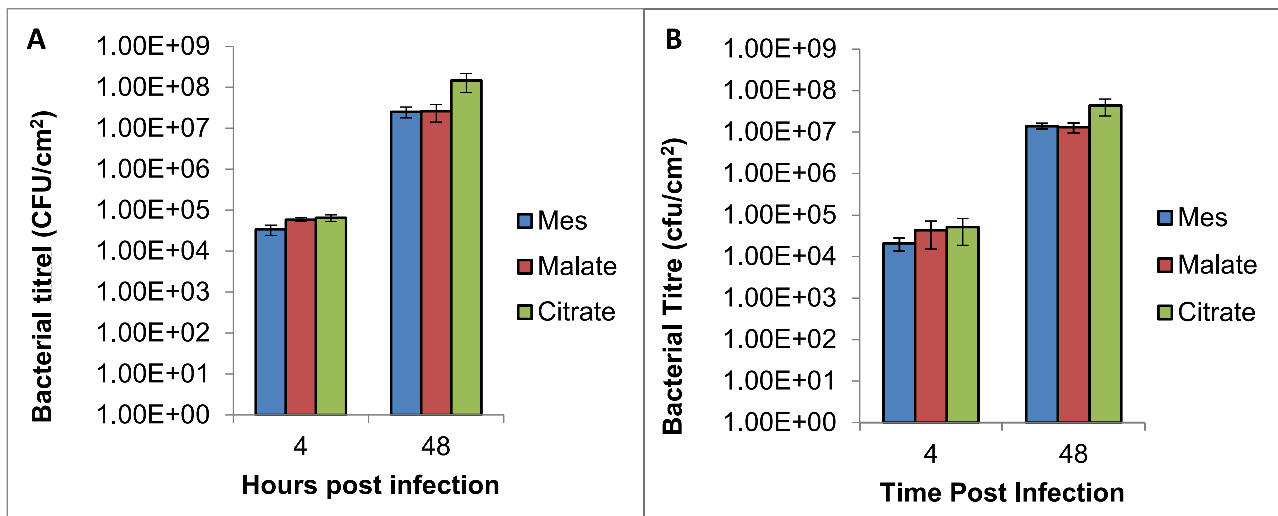

Figure 5: **Malate pre-treatment has no effect on *Pst* titres.**

Four week old *A. thaliana* plants were treated with either citrate, malate or MES prior to Pst DC3000 infection, with mean bacterial titres (n=5) calculated at 4 and 48 hpi with bars representing standard error. Figures 2A and 2B represent two independent experiments, with four performed in total.

A major limitation with the *Pst* assays was the fact that the number of biological replicates in each experiment was small (only 3-5 plants) due to the number of treatment-time point combinations being tested. Variation in *Pst* titres between the independent experiments, possibly due to damage caused by the double pressure infiltration, meant that it was not possible to pool the independent experiments to increase sample size. An alternative to pooling the data from the independent experiments was to instead look at the average fold change in *Pst* titres in response to the different metabolite treatments. This value was calculated by dividing the mean citrate or malate *Pst* titres at 48 hpi by the mean MES *Pst* titre in each of the four independent experiments (Figure 4). A one-sample t-test was then used to determine whether the average fold changes in citrate and malate in *Pst* titres were significantly different from 1 (the value predicted by the null hypothesis that the treatment had no effect). Using this statistical approach, a highly significant

difference was observed between mean *Pst* titres in citrate and MES treated plants (p=0.000965), while no such effect was seen in malate treated plants (p=0.68).

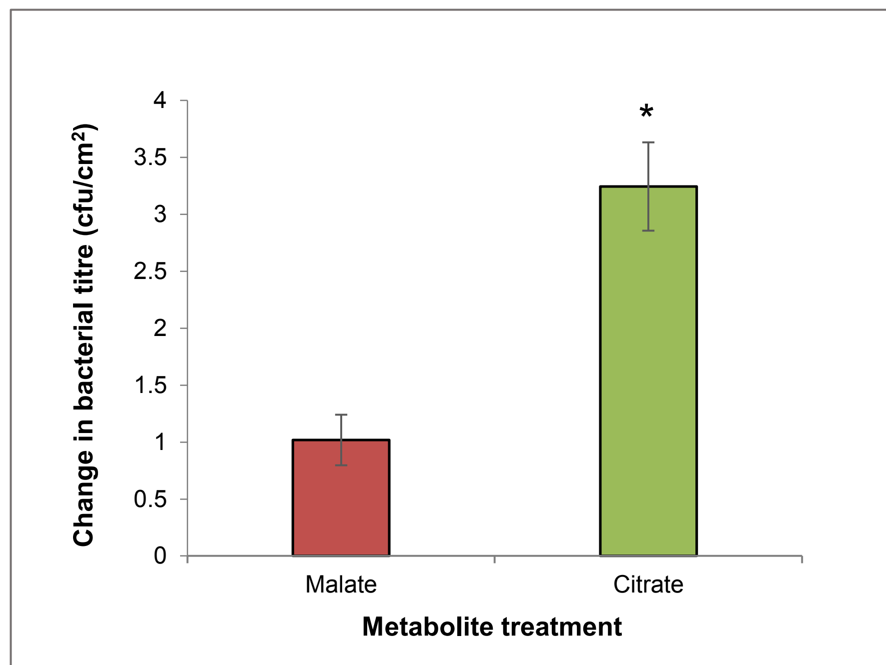

Figure 6: **Citrate but not malate pre-treatment leads to increased Pst titres in Arabidopsis.**

Mean fold increase ± SD in Pst titres (relative to MES controls) across 4 independent experiments at 48 hpi. Stars represent significant differences from value of 1 (expected value if treatment had no effect) as determined by one sample t-test

While citrate but not malate pre-treatment resulted in increased *Pst* DC3000 titres, it is possible that this effect is caused by a breakdown product of citrate rather than by citrate itself. Finkemeier *et al.*, (2013) investigated whether the changes in gene expression they observed were due to a breakdown product by investigating whether tricarballylate, a non-metabolisable isoform of citrate, could cause a similar transcriptional response in Arabidopsis. They found that tricarballylate induced the same transcriptome changes as those observed with exogenous citrate. Tricarballylate was therefore ideal to test whether the changes in bacterial titres were due to exogenous citrate itself and not a breakdown product, Plants were treated as

above, with the addition of tricarballylate (10 mM tricarballylate, in 1 mM MES pH 5.5). No significant differences in *Pst* titres were observed between citrate and tricarballylate pre-treated plants (p=0.26). Significant differences were, however, seen between citrate and MES (p=0,04), as previously observed (Figures 1 & 4), and between tricarballylate and MES (p=0,007) (Figure 5), suggesting that it is indeed citrate and not a breakdown product that is resulting in increased *Pst* titres.

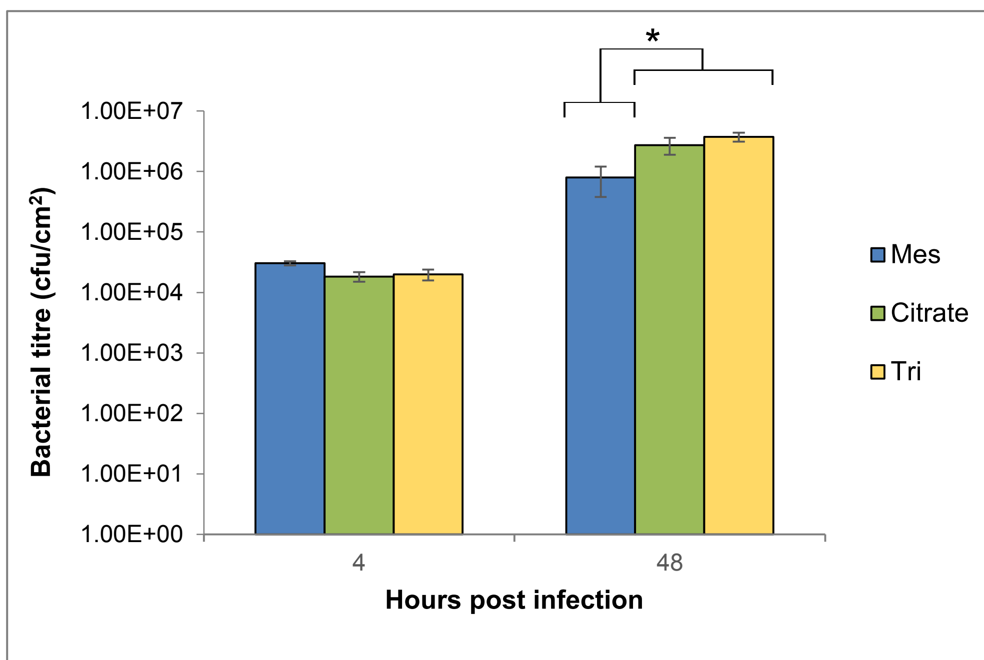

Figure 7: **Pre-treatment with tricarballylate results in increased Pst titres in Arabidopsis.**

Bars represent the standard error of the mean (n = 5). Stars represent statistical significance between bacterial titres per treatment ($P<0.05$) as determined by Student's t-test with independent variables. Data shown from one experiment.

A functional type III secretion system is required for increased Pst titres in citrate pre-treated plants

Thus far it seems that exogenous citrate is capable of increasing *Pst* growth in Arabidopsis plants. Whether this was due to exogenous citrate negatively affecting

the host immune response or positively affecting *Pst* itself was unclear. A recent study (Anderson *et al.*, 2014) proposed that citrate, as well as several other apoplastic metabolites (including succinate and shikimate) are inducers of Type Three Secretion System (TTSS) gene expression and serve as strong chemoattractant for plant pathogenic bacteria. To test whether the increased *Pst* titres observed in citrate pre-treated plants require a functional TTSS to be present in the bacteria, the experiments were repeated using the *Pst* hrpA strain which lacks a functional TTSS due to the loss of the hrpA pilus structure (Roine *et al.,* 1997). In contrast to previous experiments, there was no significant difference in *Pst* titres between control plants and those pre-treated with citrate or tricarbyllate (Figure 6). The failure of the *hrpA* mutant to multiply within the plant confirms that the TTSS is required for the observed increase in *Pst* titres observed in plants pre-treated with citrate.

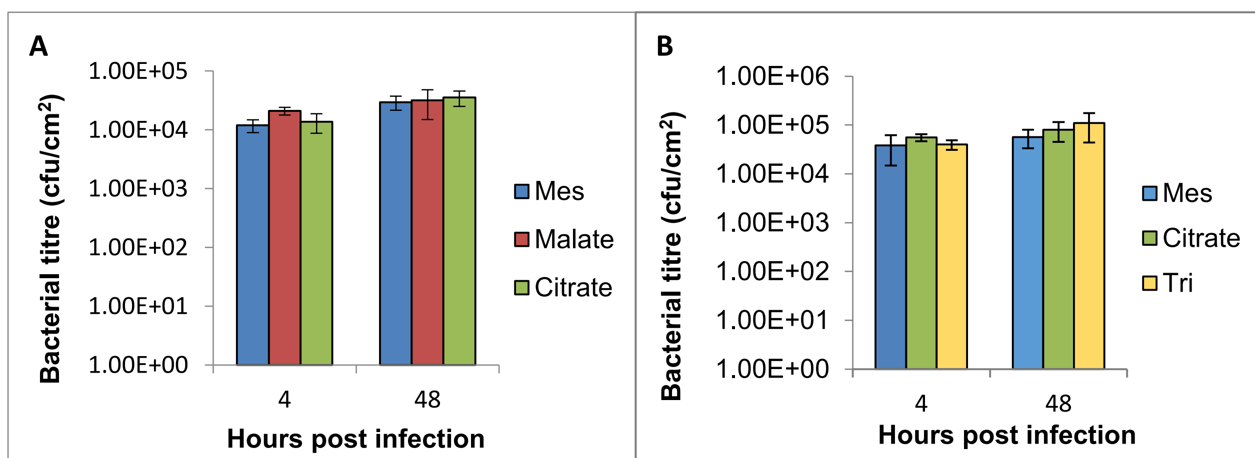

Figure 8: **A functional type III secretion system is required for increased *Pst* titres in citrate pre-treated plants**.

Mean *hrpA P. syringae* titre (with standard error, n = 5) 4 and 48 h post infection in Col-0. Data shown from two independent experiments.

Analysis of putative citrate receptors in Arabidopsis

It has been clearly demonstrated by Finkemeier *et al.* (2013) that plants are able to perceive extracellular citrate. The receptors responsible for this however, are not known. This section of work aimed to analyse several putative receptor candidates identified in Arabidopsis by sequence homology to bacterial histidine kinase citrate sensors to determine whether they are required for citrate responsiveness.

Development of an RT-PCR assay to monitor responsiveness of exogenous citrate

In order to identify possible receptors for apoplastic citrate, it was necessary to find a way to screen for citrate responsiveness in Arabidopsis. Of all the genes that were differentially expressed upon treatment with exogenous citrate, a gene of unknown function, *At1g73120* was found to be the most upregulated (24-fold) when stimulated with exogenous citrate, and expression was not detectable in control plants (Finkemeier *et al.,* 2013). The expression profile of this gene, termed *Citrate Inducible 1* (*CI1*), under various conditions was analysed using the Arabidopsis eFP browser (Winter *et al*., 2007) from the Bio-Analysis Resource (BAR) website (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). There was minimal expression found in the majority of the stress categories available, which supported the use of *CI1* as a marker gene for exogenous citrate responsiveness. For expression experiments, leaves from 4 week old plants were cut into 2-3 mm^2 pieces and vacuum infiltrated with either 1 mM MES (pH 5.5) or 10 mM citrate (1 mM MES, pH 5.5) as described by Finkemeier *et al.,* 2013). As an alternative strategy, intact leaves on four week old plants were pressure infiltrated with MES or citrate, using needleless syringes. After

6 h RNA was extracted from the samples and cDNA synthesised. PCR was performed on the cDNA, using both *CI1* and *Actin2* primers (Figure 9). *Actin2* amplification was observed in all samples barring the negative controls, confirming successful cDNA synthesis. *CI1* expression was only observed in citrate vacuum infiltrated leaf pieces, and not in the pressure infiltrated intact leaves despite flooding the apoplast. This may be due to the syringe infiltrated leaves taking longer to respond to the exogenous citrate, but this was not tested. However, the observed *CI1* expression in the vacuum infiltrated leaves confirmed the upregulation of *CI1* upon treatment with extracellular citrate as seen by Finkemeier *et al*., (2013), and supports the use of *CI1* as a marker gene for the receptiveness of extracellular citrate within plants in this study.

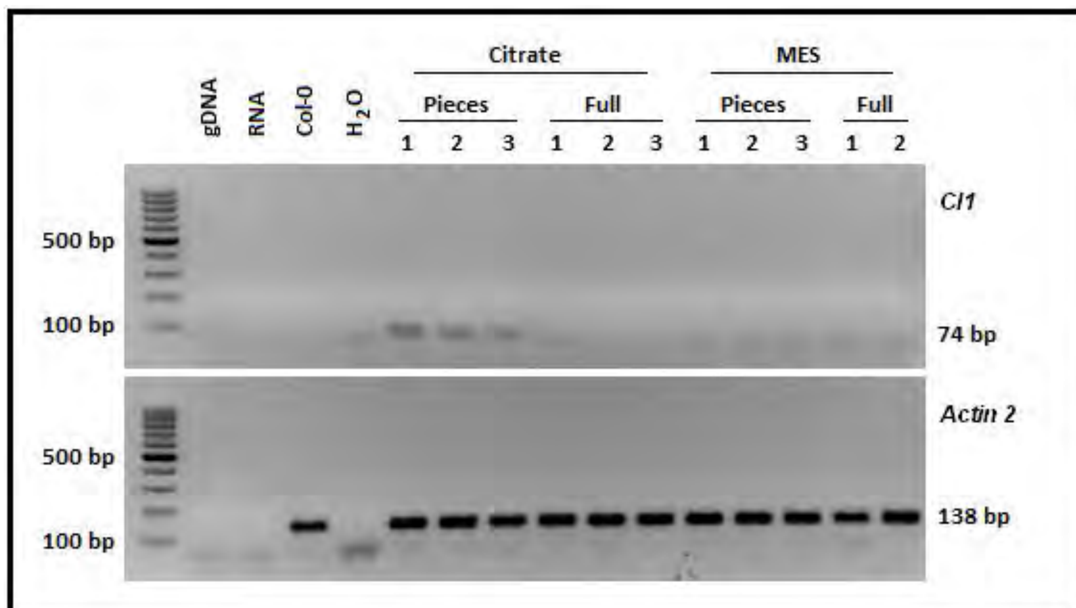

Figure 9: **CI1 expression is induced in citrate treated leaf pieces**.

cDNA from samples treated with either MES or citrate were amplified with both *Actin2* (bottom panel) and *CI1* (top panel) primers in parallel PCR reactions. Negative controls included genomic DNA (gDNA) from Col-0, RNA and water, while Col-0 cDNA was used as a positive control. The absence of a product in the gDNA lane confirms that the primers are unable to amplify products from genomic DNA.

Isolation of homozygous null lines for putative citrate receptors

Two of the four candidate genes were selected based on their modest sequence identity with the *E. coli* CitA protein (the histidine kinase citrate sensor of the two component system) in a BlastP analysis of the Arabidopsis protein database (Kasper and Bott, 2002). These proteins Histidine Kinase 1 (HK1, *At2g17820*) and Histidine Kinase 5 (HK5, *At5g10720*) belong to the 16 member histidine kinase family in Arabidopsis (Hwang *et al*., 2002). HK1 has been shown to localise to the plasma membrane (Ceasar *et al*., 2011), the most likely location for a receptor involved in apoplastic citrate sensing, while HK5 localises to both cytoplasm and plasma membrane (Desikan *et al*., 2008). Both proteins appear to play multiple roles in plants. HK1 has previously been shown to act as a sensor for both drought and salt stress (Urao *et al*., 1999), while HK5 has been implicated in both abiotic and biotic stress signalling (Pham *et al*., 2012). An additional role as a citrate sensor is thus plausible. A third *Histidine Kinase 2* (*HK2*, At5g35750) which is involved in cytokinin responsiveness (Higuchi *et al*., 2004) was selected as a final HK candidate gene as it is also predicted to localise to the plasma membrane. The final candidate gene, was chosen due to its predicted role in the synthesis of citrate and predicted localisation. An isoform (At3g58750) of *Citrate Synthase 2* (*CS2*), an enzyme involved in the catalysis of acetyl coenzyme A (acetyl CoA) and oxaloacetic acid to form citric acid, has been predicted to localise to the apoplast of *A. thaliana* (Slabas *et al*., 2004). As Hexokinase, a key enzyme is the synthesis of glucose, has also been shown to be a receptor for glucose and is involved in the glucose signalling pathway (Jang *et al*., 1997), it was decided to test whether CS2 is involved in the responsiveness of exogenous citrate, given its predicted extracellular localisation.

In order to determine whether any of these candidate genes play a role in exogenous citrate responsiveness, it was necessary to obtain mutants in which the genes of interest were silenced. This was done by obtaining T-DNA insert mutants for each candidate gene from NASC (http://arabidopsis.info). As the creation of T-DNA mutants is prone to multiple insertion events, it was decided to attempt to obtain two independent T-DNA insert lines per gene. This would provide a greater degree of certainty that any observed phenotype was due to the silencing of the gene of interest and not due to random secondary insertion events.

To isolate individuals homozygous for T-DNA inserts, gDNA was extracted from a number of individuals from each T-DNA insert line (minimum 5 plants) as well as from Col 0 plants. PCR genotyping was performed using gene specific primer pairs (LP + RP) and insert specific primer pairs (BP + RP) in separate reactions. Plants homozygous for the wild type allele will display a PCR product in only the LP + RP primer reaction, while those homozygous for the T-DNA insert will display a product with the BP + RP primer pair only (Figure 10). Plants that were heterozygous for the T-DNA insert will display a product with both sets of primers. If only heterozygous individuals were detected then these plants were allowed to self-fertilise and the resulting progeny were screened for homozygous individuals.

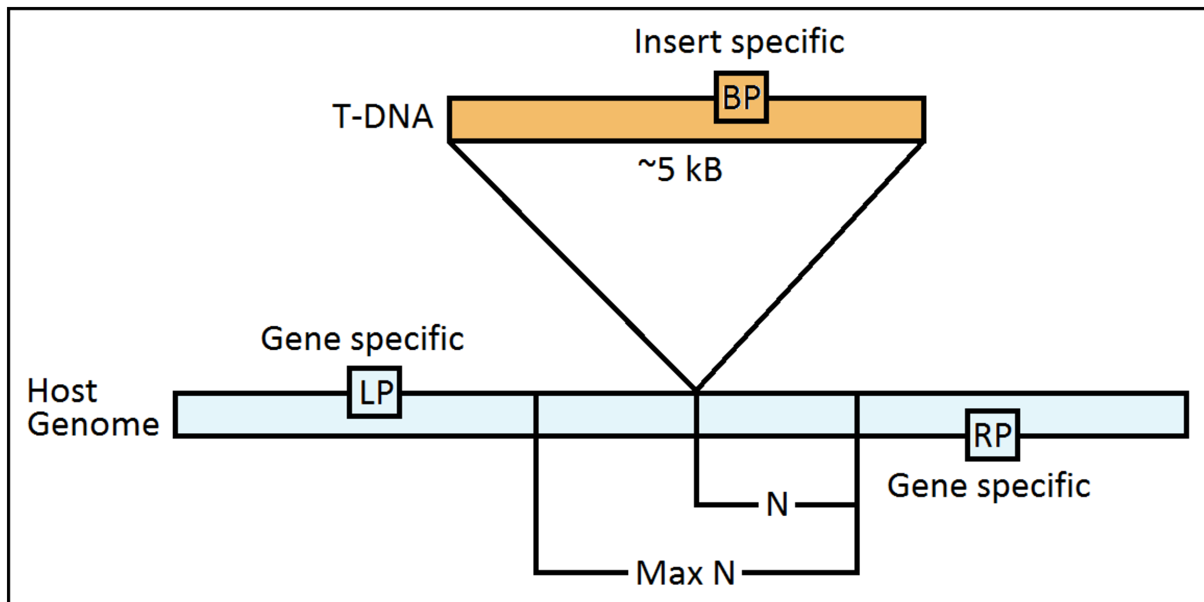

Figure 10: **PCR based genotyping of T-DNA insertion mutants.**

Gene specific primers (LP and RP respectively) are designed to amplify wild type alleles and flank the predicted T-DNA insertion site. The T-DNA insert specific border primer (BP) is situated on the left border of the T-DNA insert and is able to amplify a product with the gene specific RP only in the presence of a T-DNA insert. Max N represents the approximate number of base pairs expected in a PCR product from amplification with the gene specific primers in the absence of an insert (LP + RP), while the N is the approximate number of base pairs amplified with the insert specific primer and right gene specific primer (BP + RP).

In the putative *HK1* T-DNA insert lines (Figure 11), *hk1-1* DNA samples consistently gave a product only with the gene specific primers (Figure 11A) while plant samples from the *hk1-2* line gave a product only in the T-DNA insert specific PCR (Figure 11B). The absence of T-DNA amplification from the *hk1-1* plant line indicates it is homozygous wild type for the *HK1* allele, while the absence of WT amplification in the *hk1-2* plant line indicates it is homozygous for the T-DNA insert.

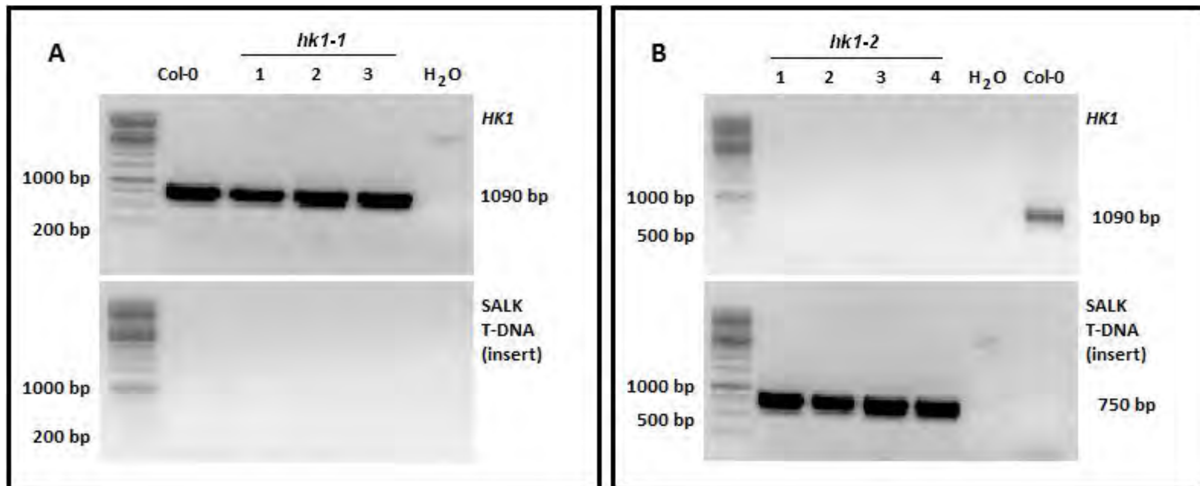

Figure 11: **Identification of homozygous T-DNA insert lines for *HK1*.**

PCR amplification of DNA extracted from Col-0 and the two putative *HK1* T-DNA mutant lines. DNA was amplified using wither gene specific primers (*HK1*-LP + *HK1*-RP) or T-DNA insert specific primers (SALK LBb1.3 and *HK1*-RP) with Col-0 as a positive control for wild-type gDNA, and water as a negative control.

Identification of *HK2*, *HK5* and *CS2* plants homozygous for the T-DNA insert were carried out in the same manner as for *HK1*. In the *HK2* T-DNA insert line *hk2-1* a product was observed in all DNA samples using the T-DNA primer set, with no PCR products observed in the WT primer reaction (Figure 12A), confirming that this line is homozygous for the T-DNA insertion. In contrast, DNA from the *hk2-2* plant line generated PCR products in both the gene and T-DNA insert specific reactions indicating that all individuals tested were heterozygous (Figure 12B). Although these heterozygous individuals were self-fertilised, no homozygous progeny were detected in the next generation (data not shown).

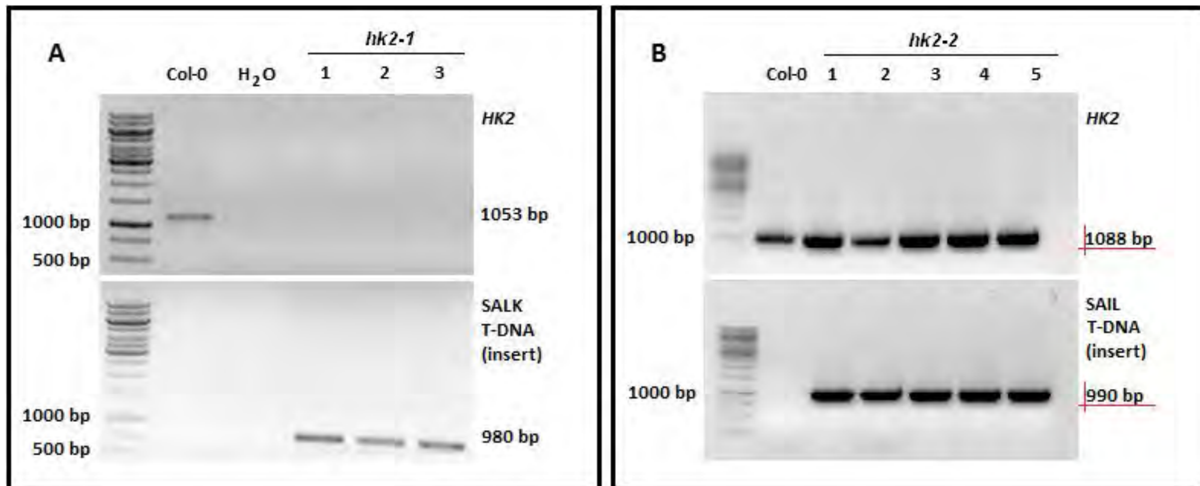

Figure 12: **Identification of homozygous T-DNA insert lines for *HK2*.**

PCR amplification of DNA extracted from Col 0 and plants from each of the two *HK2* T-DNA mutant lines. DNA was amplified using either gene specific primers (*HK2*-LP + *HK2*-RP, upper panel) or T-DNA insert specific primers (either SALK LBb1.3 (A) or SAIL LB3 (B) and *HK2*-RP, lower panel) with Col 0 as a positive control for wild-type gene structure, and water as a negative control in A. The water control was absent in the *HK2*-1 amplification.

For *HK5* amplification of WT PCR product was seen in all DNA samples from the *hk5-1* T-DNA insert line, while amplification from the T-DNA primer set was observed in three of the eight samples (Figure 13A). In order to identify a homozygous individual in the next generation, 20 individuals were screened from the progeny of a self-fertilizing heterozygote. Of the individuals screened, none of the offspring were identified as homozygous, with all being either homozygous wild type or heterozygous. In contrast, in the *hk5-2* plant line, amplification was only observed with the T-DNA primer set (Figure 13B). This indicated that the *hk5-1* plant line possesses both WT and heterozygous individuals, while the *hk5-2* plant line is homozygous for the T-DNA insert.

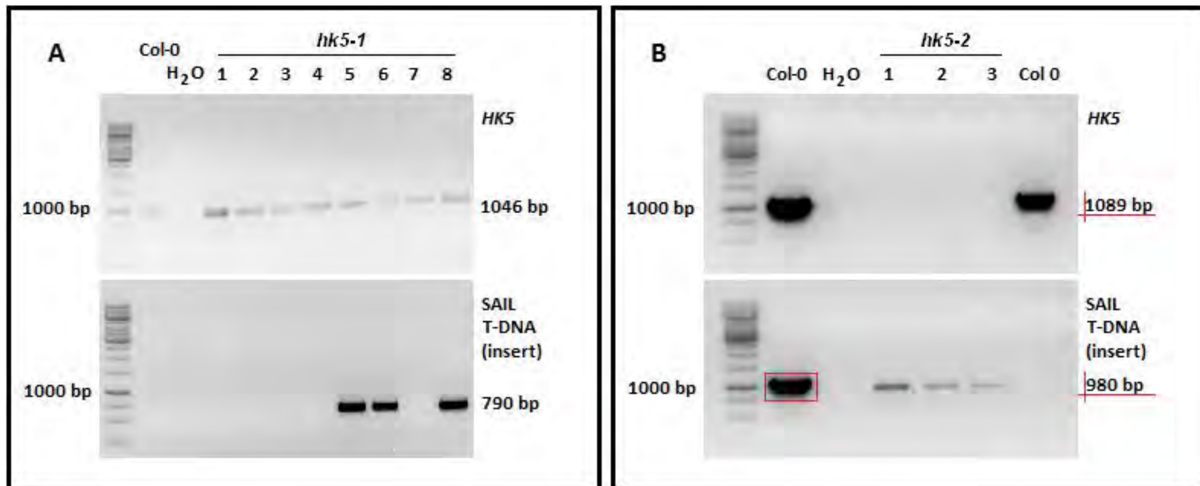

Figure 13: **Identification of homozygous T-DNA insert lines for *HK5*.**

PCR amplification of DNA extracted from Col 0 and plants from each of the two *HK5* T-DNA mutant lines. DNA was amplified using wither gene specific primers (*HK5*-LP + *HK5*-RP, top panel) or T-DNA insert specific primers (either SALK LBb1.3 (A) or SAIL LB3 (B) and *HK5*-RP, bottom panel) with Col 0 as a positive control for wild-type allele structure, and water as a negative control. The red box in B represents Col-0 DNA that was amplified using the same WT primers as in the top panel.

In both *CS2* T-DNA plant lines PCR amplification was only seen with insert specific primers (Figures 14A and 14B) indicating that both plant lines obtained were homozygous for the T-DNA insert.

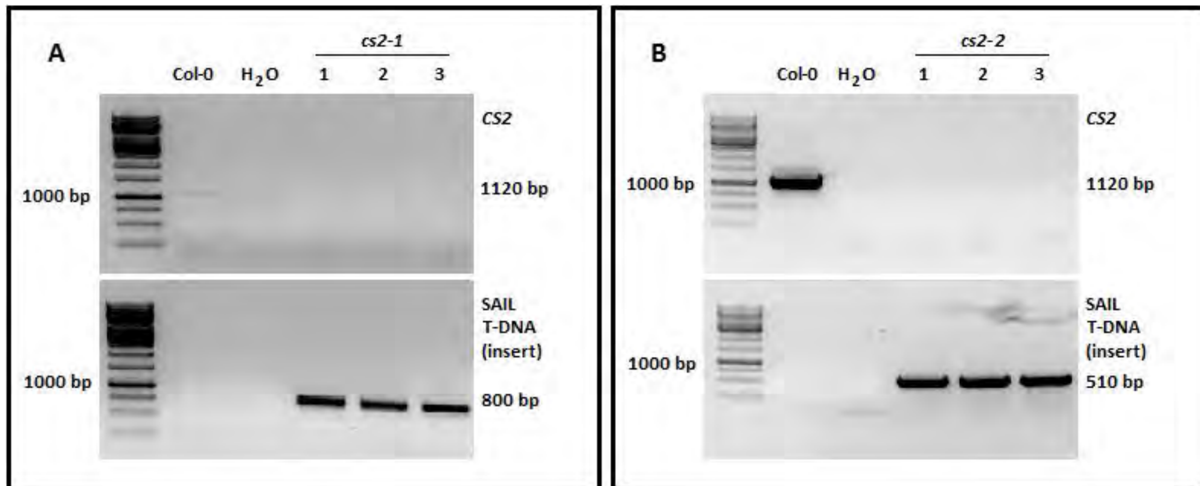

Figure 14: **Identification of homozygous T-DNA insert lines for *CS2*.**

PCR amplification of DNA extracted from Col 0 and plants from each of the two *CS2* T-DNA mutant lines. DNA was amplified using either gene specific primers (*CS2*-LP + *CS2*-RP, top panel) or T-DNA insert specific primers (SALK LBb1.3 and *CS2*-RP, bottom panel) with Col 0 as a positive control for wild-type allele, and water as a negative control.

The progeny of plant lines identified as homozygous for the T-DNA insert through PCR genotyping were screened on selective media to confirm their homozygous genotype. SALK T-DNA lines contain the kanamycin resistant gene *NPTII* while SAIL lines display DL-Phosphinothricin resistance.

Seed was collected from self-fertilised individuals identified as homozygous insertion mutants for their genes of interest. Seed from SALK plants was grown on half-strength MS agar containing kanamycin (20ug/ml) for one week. Plant growth was only observed only on control plates without kanamycin, indicating that although the seeds were viable, they were no longer expressing the *NPTII* gene. As gene silencing of selection markers has been shown to occur after several filial generations (Ostergaard and Yanofsky, 2004), this likely explains why the homozygous mutant plants were not displaying the expected resistance phenotype.

As the only identified homozygous SAIL line, seed obtained from the *hk5-2* insertion line was planted on 1:1 peat and vermiculite soil, with Col-0 plants as a negative control. Each pot was liberally sprayed with the commercial herbicide BASTA (DL-Phosphinothricin) at a concentration of 30mg/l when plants were 2 weeks of age, at three time-points (0, 24 and 72 hours after the first application) (Figure 15). A graph of survival was plotted from the number of surviving plants observed at 0, 24, 48 and 72 hours post initial treatment. The survival profile of the *hk5-2* plants upon BASTA exposure corresponded to that of the positive control Arabidopsis plants carrying empty pFAST-G02 vector (homozygous), confirming that the plants were homozygous for the T-DNA insert.

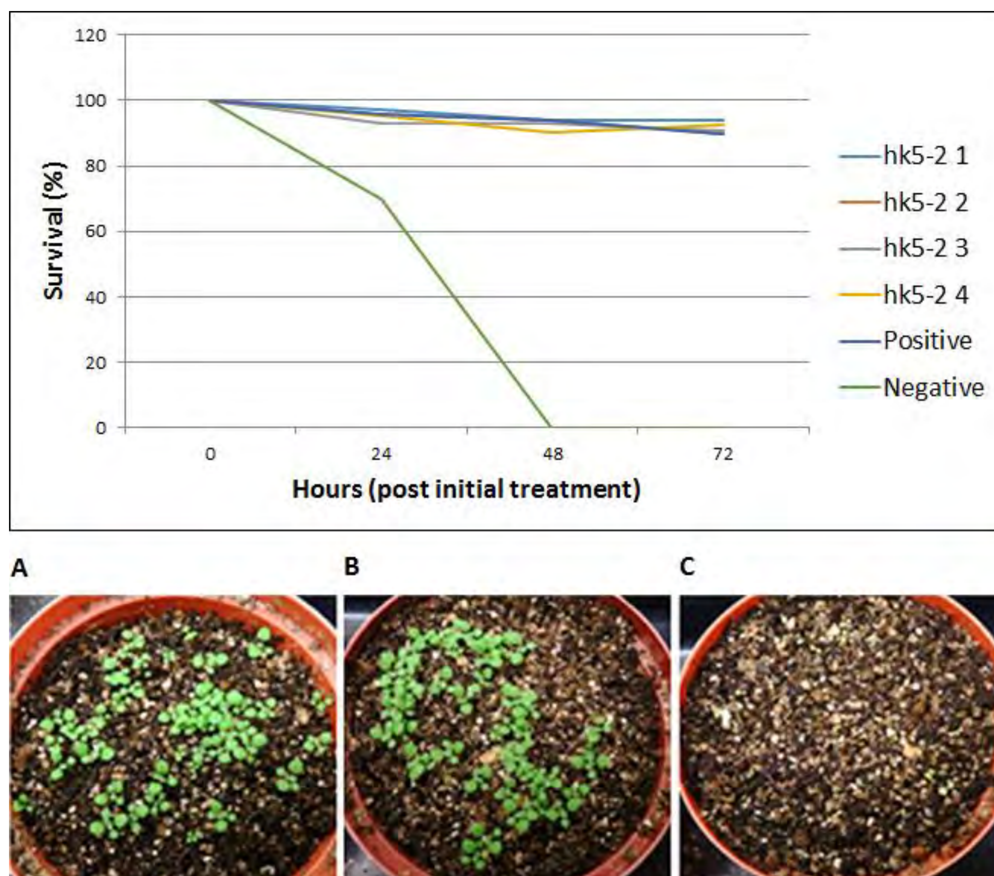

Figure 15: **DL- Phosphinothricin selection confirms homozygosity of the *hk5-2* mutant line.**

Survival over time is shown 0, 24, 48 and 72 h post initial DL- Phosphinothricin treatment. Repeat treatments occurred at 24 and 72 h respectively. Observed growth in the positive control (A), negative control (Col-0) (B) and *hk5-2* mutant line (C) after 72 hours.

Confirmation of genomic location of T-DNA inserts in homozygous lines

Sequencing of the purified PCR insert specific products for all lines was carried out to confirm the actual genomic location of the T-DNA inserts. This revealed that the actual T-DNA insertion sites are all present within 300 bp of the predicted insertion sites (http://signal.salk.edu/cgi-bin/tdnaexpress) (Figure 16). In the *HK1*, *HK2* and *CS2* T-DNA insert plant lines, all inserts were located in the 5' upstream region, either in the 5' UTR (as seen in the *cs2-2* plant line) or further upstream. In the *HK5* plant lines the insertion site occurs within the 10th exon of the gene. The insert of DNA in the upstream region of the GOIs may suppress gene expression by blocking transcription, while disruption of the gene itself (as seen in the HK5 insertion lines) may result in gene silencing through formation of a truncated or inactive protein.

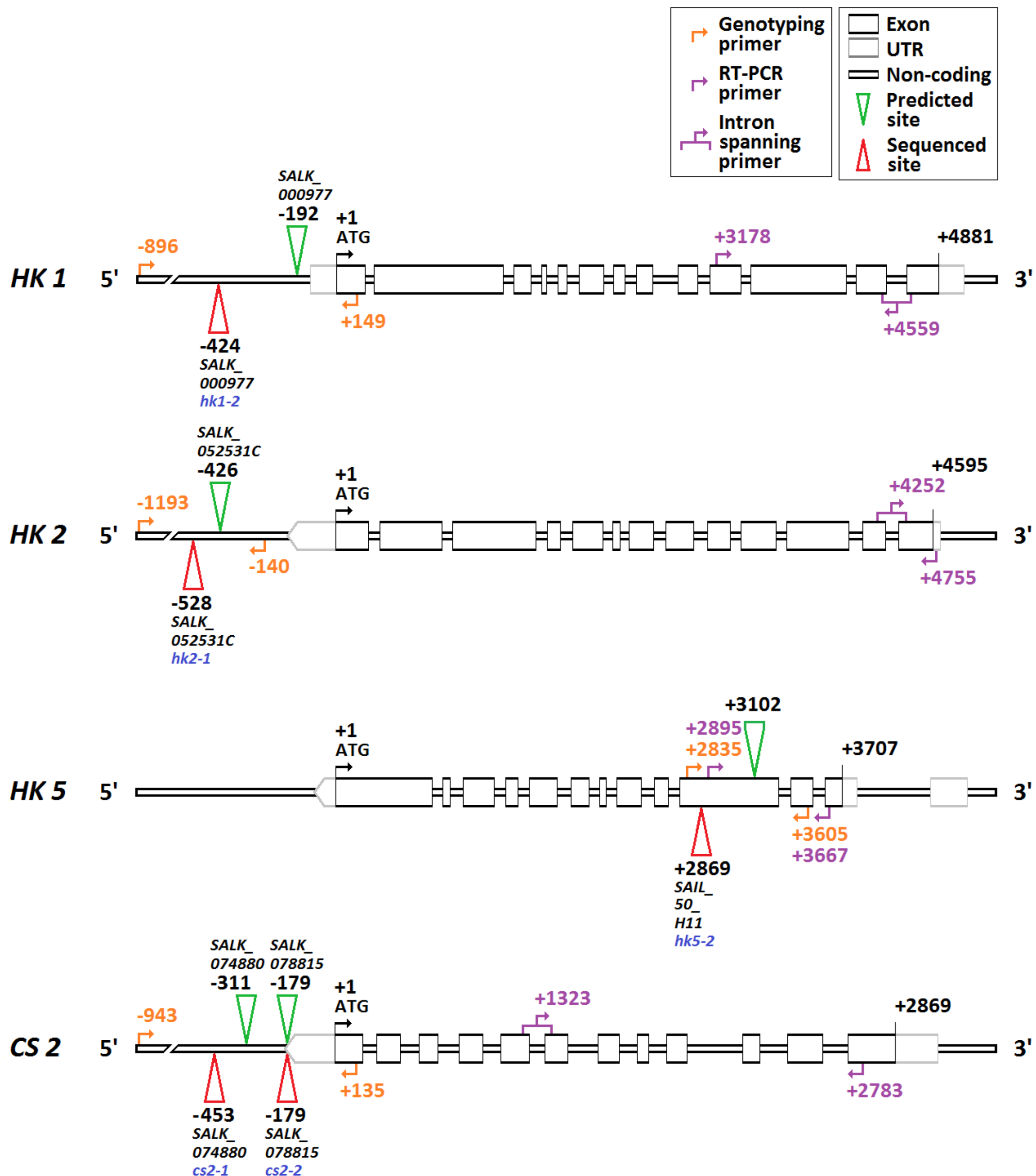

Figure 16: **Predicted versus actual insertion sites for homozygous T-DNA lines used in this study.**

Insert sites predicted by the SALK T-DNA database are indicated in green and those determined by DNA sequencing are shown in red. Exons are represented by black blocks, UTR by grey and non-coding DNA by the thinner black sections. Gene loci are numbered in relation to the start (ATG) codon (+1) of that specific gene. Each insert is labelled according to insert name in black, and according to its mutant name in blue underneath each sequenced insert. Genotyping and knock-out primers are shown in orange and purple respectively.

RT-PCR Confirmation of gene knock down in T-DNA mutant lines

Although homozygous T-DNA insertion lines were identified for each gene of interest, it was necessary to check whether they were indeed null mutants. To determine whether expression of the gene of interest was abolished in these plant lines, RT-PCR was performed on RNA extracted from 2 week old seedlings with gene specific primers downstream of the confirmed T-DNA insertion sites (Figure 16). Successful amplification of PCR products for all four genes was observed with Col-0 cDNA, while no GOI products were seen in the *hk1-2*, *hk2-1* and *hk5-2* samples, confirming that they were true knock-out mutants (Figure 17). However, in the *CS2* insertion lines, amplification was observed in all samples. The presence of product in the *CS2* homozygous mutants suggests that the location of the *CS2* insertion sites in the u*pst*ream region of the CS2 gene is not sufficient to abolish transcription of this gene.

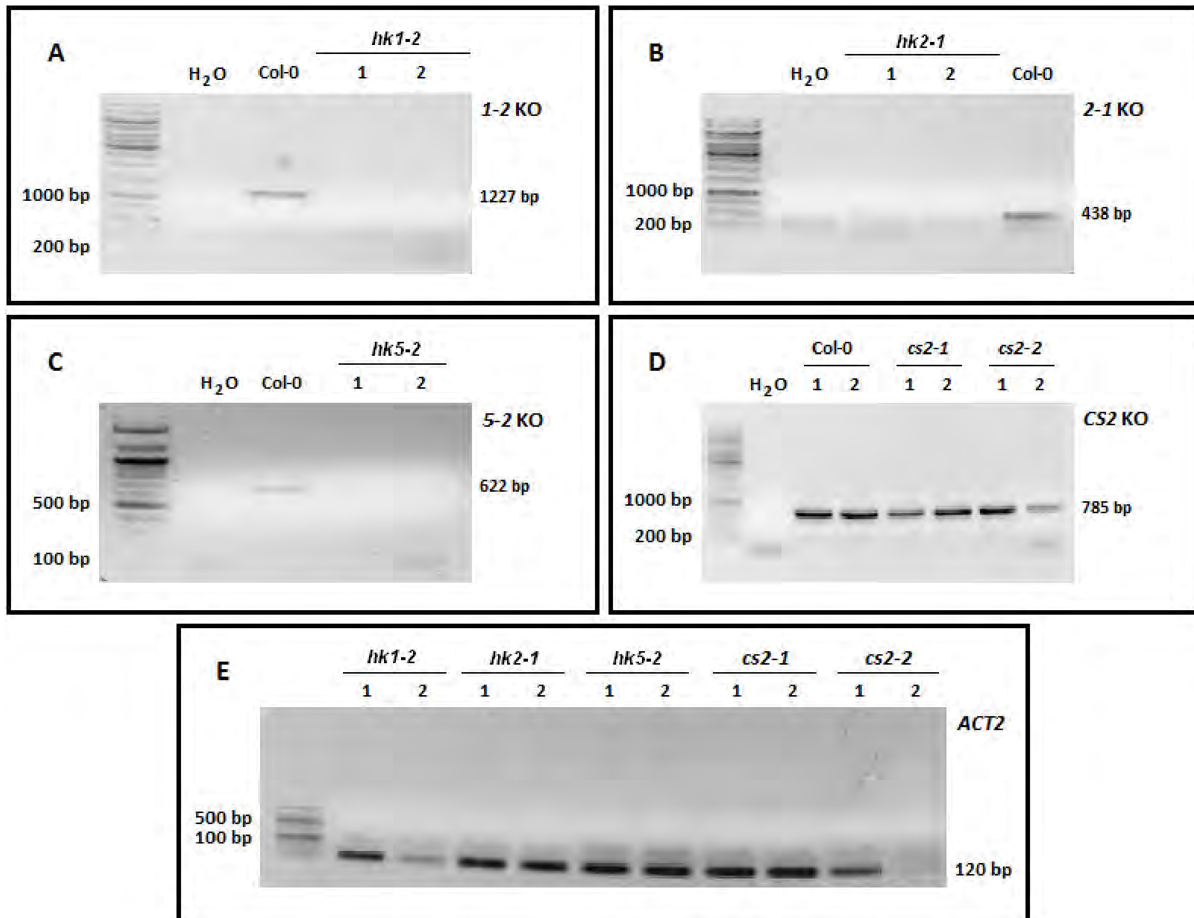

Figure 17: **RT-PCR analysis of GOI expression in homozygous T-DNA insert lines confirms *hk1-2*, *hk2-1* and *hk5-2* are true knock out mutants.**

PCR amplification of cDNA from 2 week old seedlings. Controls include water as a negative and Col-0 as a positive for the gene specific primers. Figures A, B, C and D represent *hk1-2, hk2-1, hk5-2* and *CS2* mutant lines respectively. Figure E shows *ACT2* products for all mutant samples used in A, B, C and D with the water control absent.

Analysis of *CI1* expression in null mutants

The ability of the null mutants *hk1-2*, *hk2-1* and *hk5-2* to perceive exogenous citrate was assayed by RT-PCR analysis of the *CI1* gene. As described above, expression of this gene is observed in Col-0 plants following vacuum infiltrated with citrate and not in control plants treated with MES. If any of the candidate genes is required for citrate responsiveness within the plant then induction of *CI1* should be abolished in a null mutant.

Leaves from 4 week old plants were cut into 2-3 mm^2 pieces and vacuum infiltrated with either 1 mM MES (pH 5.5) or 10 mM citrate (1 mM MES, pH 5.5). RNA was then extracted and used to generate cDNA which was used as template in a standard PCR. *CI1* expression was evident in all three null mutants tested (Figure 18), suggesting that these genes are not required for citrate responsiveness in Arabidopsis, or alternatively if involved play redundant roles in this process.

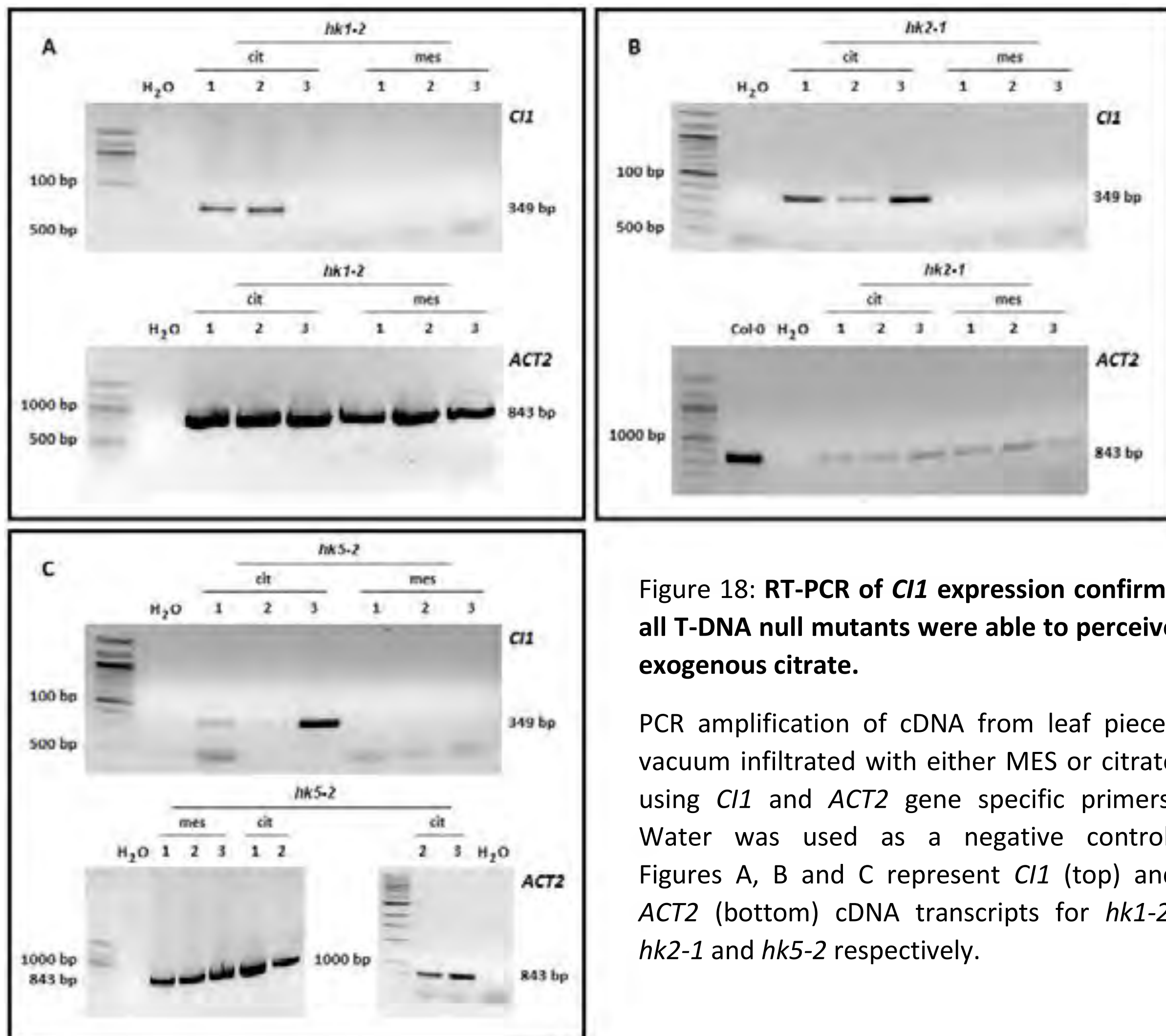

Figure 18: **RT-PCR of *CI1* expression confirms all T-DNA null mutants were able to perceive exogenous citrate.**

PCR amplification of cDNA from leaf pieces vacuum infiltrated with either MES or citrate using *CI1* and *ACT2* gene specific primers. Water was used as a negative control. Figures A, B and C represent *CI1* (top) and *ACT2* (bottom) cDNA transcripts for *hk1-2, hk2-1* and *hk5-2* respectively.

Chapter 4: Discussion and Conclusion

Phytopathogens that proliferate and grow within the host apoplast are required to suppress the immune system of their hosts or else face restricted growth or death. In order to suppress the immune response that they induce in their hosts, pathogens have evolved an array of molecular mechanisms to negate both PTI and ETI (Macho *et al*., 2010; Liu *et al*., 2011). In bacterial pathogens, including *Pst* DC3000, the secretion of effectors into the host cells via the type III secretion system enables the direct suppression of immune responses, allowing colonisation within the host (Macho *et al*., 2010).

While undoubtedly important, effectors are not the only mechanism by which pathogens subvert the host immune response. Pathogens also produce phytotoxins, including coronatine which plays an important role in overcoming stomatal-based defence (Melotto *et al*., 2006). The focus of this thesis was to examine whether the tri-carboxylic acid citrate, a metabolite of the TCA cycle, might also play a role in pathogen suppression of the immune response. Citrate is secreted into the apoplast by both the host and bacterial pathogens including *Pst* DC3000 (Miethke *et al*., 2006) and has previously been shown to act as a powerful chemo-attractant for *Pst* DC3000, enabling the pathogen to recognise that it is within a potential host (Cuppels, 1988). Once the pathogen has initiated contact with plant cells, exogenous citrate also induces genes associated with the T3SS (Anderson *et al*., 2014). This dual function strongly enhances the virulence of infecting *Pst* DC3000. In addition, citrate itself may also have a regulatory function on *Arabidopsis* defence gene expression even in the absence of a pathogen (Finkemeier *et al*., 2013). This transcriptional regulation was found to be specific to citrate, with malate being the

only other TCA metabolite to induce differential transcript levels, albeit to a far lesser extent than citrate. That the transcriptome profile after citrate treatment was most similar to that seen in response to *Pst* DC3000 and flg22 treatment suggest that exogenous citrate could play a role in pathogen-host interactions.

In this study two approaches were taken to examine the biological role played apoplastic citrate. Firstly, it was investigated whether pre-treatment of Arabidopsis with citrate influences the outcome of the plant-pathogen interactions. Secondly, once the ability of Arabidopsis to respond to exogenous citrate was confirmed, candidate proteins were analysed in order to attempt to identify possible citrate sensors.

Citrate pre-treatment leads to increased *Pst* titres in Arabidopsis

Initial results utilizing the Arabidopsis-*Pst* Pathosystem, showed higher bacterial titres 48 hpi in plants pre-treated with exogenous citrate (10 mM) 24 h prior to infection with *Pst* DC3000 than those treated with the MES control (Figure 1). This increase in bacterial titre was not due to any osmotic effect, as treatment with 10 mM KCl did not result in the increased *Pst* DC3000 levels (Figure 2), nor was it due to any pH effect, as the citrate was buffered with MES to pH 5.5. No such increase in bacterial titre was observed in response to another TCA cycle metabolite, malate (Figure 4), which had a much smaller effect on the transcriptome in Arabidopsis (Finkemeier *et al.*, 2013). That pre-treatment with the non-metabolisable citrate analogue tricarballylate resulted in a similar increase in bacterial numbers as did citrate (Figure 5) suggests that it is citrate itself causing the observed increase in bacterial titres rather than any of its breakdown products.

Given the known role of citrate in inducing T3SS gene expression, whether citrate pre-treatment allowed increased growth of the T3SS mutant *hrpA* was investigated. This strain possesses a mutation in the gene required for the hrpA pilus structure, resulting in a non-functional T3SS (Roine *et al.,* 1997). In contrast to assays using the *Pst* DC3000 virulent strain (Figures 1-5), no significant increase in *Pst* titres at 48 hpi was observed in citrate pre-treated plants infected with the *hrpA* mutant (Figure 6). This was despite the fact that genes known to be involved in PTI responses, including *WRKY11* and *FRK1*, had been identified as downregulated after citrate treatment (Finkemeier *et al*., 2013), which led to the hypothesis that this might allow successful colonisation of the host by T3SS mutants. The findings contradict this hypothesis, suggesting instead that the extent of downregulation of PTI by citrate is not sufficient to allow the *hrpA* mutant to proliferate.

The results obtained in this study indicate that citrate pre-treatment of Arabidopsis leads to increased *Pst* DC3000 titres 48 hpi, but only if the bacteria have a functional TTSS. It is thus unclear at the present time whether increased apoplastic citrate concentrations cause this through a suppressive effect on the host immune response, an enhanced induction of the TTSS system in *Pst,* or a combination of both. Quantitative analysis of host PAMP-triggered defence genes including *WRKY11* and *FRK1* and *Pst* TTSS genes following infiltration of citrate pre-treated and control plants with *Pst* would greatly aid in determining the mechanism by which apoplastic citrate exerts its effect on *Pst* titres It would also be interesting to determine whether this modulation of the host immune response extends to ETI. This could be investigated by repeating the experiments with *Pst* DC3000 strains possessing specific Avr effectors e.g. AvrB or AvrRpm1 which are detected by the RPM1

resistance protein in Arabidopsis, which guards RIN4 the target of these effectors (Mackey *et al*., 2003)

<u>*HK1, 2* and *5* are not required for citrate responsiveness in Arabidopsis</u>

It is clear that plants are able to perceive exogenous citrate (Finkemeier *et al*., 2013), but the mechanism by which they do so is unknown. The bacterium *E.coli* is capable of detecting exogenous citrate through the membrane-bound sensory histidine kinase CitA (Payne and Neilands, 1988). Using a homology search in Arabidopsis, two histidine kinases (*HK 1* and *5*) were identified that shared a degree of amino acid sequence similarity with the C terminus of CitA. These proteins both belonged to the HK superfamily, from which a third *HK* gene was selected, *HK2*. The final candidate gene, an isoform of *Citrate Synthase 2* which has been detected in apoplastic fractions (Slabas *et al*., 2004), was selected to investigate whether it might act as a citrate sensor, analogous to the role performed by hexokinase in glucose signalling. Having identified candidates, a marker for citrate responsiveness was required in order to analyse their possible roles in citrate recognition.

It had been found that *CI1*, a gene coding for a protein of unknown function, was one of the two most strongly up-regulated genes after treatment with citrate, and that its up-regulation was citrate specific (Finkemeier *et al*., 2013). In addition, *CI1* is not expressed within the plant under normal conditions (Figures S1 and S2, Supplementary Data). This made it an ideal marker for the responsiveness of the plant to exogenous citrate.

CI1 expression in Col-0 Arabidopsis in citrate and MES treated leaf slices was confirmed as only occurring in leaf samples treated with citrate (Figure 7). Having

confirmed *CI1* as a marker gene for citrate responsiveness, T-DNA insert lines for the four candidate genes were screened to isolate homozygous individuals. One homozygous individual was identified per *HK* gene (*hk1-2*, *hk2-1* and *hk5-2* respectively) (Figures 9-11), while both *cs2* mutant lines were found to be homozygous for the insert (Figure 12). However, RT-PCR analysis revealed that only the three *hk* homozygous T-DNA lines were null mutants, whereas full length *CS2* transcripts were detected in both *cs2* homozygous T-DNA lines (Figure 15). *CI1* expression was analysed using end-point RT-PCR in the three *hk* mutants, and the gene was found to be induced in response to exogenous citrate in all three lines. These results indicate that these genes are not required for the responsiveness of the plant to exogenous citrate. However it is possible that they may play a redundant role in this process. The generation of double or triple mutants in these genes would be required to investigate this possibility. Another approach to identify possible citrate receptors would be through the use of a citrate affinity column, which could be used to pull down citrate binding proteins from plant membrane fractions.

The results from this project confirm that plants are capable of detecting exogenous citrate although the mechanisms for this responsiveness are still unclear. It has also been shown that pre-treatment with citrate influences the outcome of the *Arabidopsis*-*Pst* interaction. It would be interesting to dissect the mechanisms involved in this to determine whether it is due to suppression of host defence genes, enhanced induction of the T3SS or both. This would help determine if citrate is truly acting as a metabolic signal to repress defence gene expression during pathogen attack. Finally, by comparing the citrate concentration secreted by *Pst* DC3000 during infection to the concentration of plant generated citrate present in the apoplast

prior to infection, it may be possible to identify the originator of the citrate signal as *Pst* or the host itself.

Chapter 5: References

Abramovitch, R.B. and Martin, G.B., 2004. Strategies used by bacterial pathogens to suppress plant defenses. *Current opinion in plant biology. 7*:356-364.

Anderson, J.C., Wan, Y., Kim, Y.M., Pasa-Tolic, L., Metz, T.O. and Peck, S.C., 2014. Decreased abundance of type III secretion system-inducing signals in Arabidopsis mkp1 enhances resistance against *Pseudomonas syringae*. *Proceedings of the National Academy of Sciences.* 111:6846-6851.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M. and Sheen, J., 2002. MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature.* 415:977-983.

Bender, C.L., Alarcón-Chaidez, F. and Gross, D.C., 1999. *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiology and Molecular Biology Reviews*. 63:266-292.

Block, A., Li, G., Fu, Z.Q. and Alfano, J.R., 2008. Phytopathogen type III effector weaponry and their plant targets. *Current opinion in plant biology.* 11:396-403.

Bouche, N. and Fromm, H., 2004. GABA in plants: just a metabolite? *Trends in plant science*. 9:110-115.

Bradley, D.J., Kjellbom, P. and Lamb, C.J., 1992. Elicitor-and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell*. 70:21-30.

Brooks, D.M., Bender, C.L. and Kunkel, B.N., 2005. The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. *Molecular Plant Pathology*. 6:629-639.

Brown, J.C., 1978. Mechanism of iron uptake by plants. *Plant, Cell & Environment*. 1:249-257.

Caesar, K., Thamm, A.M., Witthöft, J., Elgass, K., Huppenberger, P., Grefen, C., Horak, J. and Harter, K., 2011. Evidence for the localization of the Arabidopsis cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. *Journal of Experimental Botany*. 62:5571-5580.

Chen, Z., Agnew, J.L., Cohen, J.D., He, P., Shan, L., Sheen, J. and Kunkel, B.N., 2007. *Pseudomonas syringae* type III effector AvrRpt2 alters *Arabidopsis thaliana* auxin physiology. *Proceedings of the National Academy of Sciences.* 104:20131-20136.

Chisholm, S.T., Coaker, G., Day, B. and Staskawicz, B.J., 2006. Host-microbe interactions: shaping the evolution of the plant immune response.*Cell*. 124:803-814.

Chou, H.M., Bundock, N., Rolfe, S.A. and Scholes, J.D., 2000. Infection of q *Arabidopsis thaliana* leaves with *Albugo candida* (white blister rust) causes a reprogramming of host metabolism. *Molecular Plant Pathology*. 1:99-113.

Collier, S.M. and Moffett, P., 2009. NB-LRRs work a "bait and switch" on pathogens. *Trends in plant science.* 14:521-529.

Cui, J., Bahrami, A.K., Pringle, E.G., Hernandez-Guzman, G., Bender, C.L., Pierce, N.E. and Ausubel, F.M., 2005. *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. *Proceedings of the National Academy of Sciences of the United States of America*. 102:1791-1796.

Cunnac, S., Lindeberg, M. and Collmer, A., 2009. *Pseudomonas syringae* type III secretion system effectors: repertoires in search of functions. *Current opinion in microbiology*. 12:53-60.

Cuppels, D.A., 1988. Chemotaxis by *Pseudomonas syringae pv. tomato*. *Applied and environmental microbiology*. 54:629-632.

Desikan, R., Horák, J., Chaban, C., Mira-Rodado, V., Witthöft, J., Elgass, K., Grefen, C., Cheung, M.K., Meixner, A.J., Hooley, R. and Neill, S.J., 2008. The histidine kinase AHK5 integrates endogenous and environmental signals in Arabidopsis guard cells. *PLoS One.* 3:e2491.

Deslandes, L., Olivier, J., Peeters, N., Feng, D.X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S. and Marco, Y., 2003. Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proceedings of the National Academy of Sciences*. 100:8024-8029.

Dodds, P.N. and Rathjen, J.P., 2010. Plant immunity: towards an integrated view of plant–pathogen interactions. *Nature Reviews Genetics*. 11:539-548.

Dodds, P.N., Lawrence, G.J., Catanzariti, A.M., Teh, T., Wang, C.I., Ayliffe, M.A., Kobe, B. and Ellis, J.G., 2006. Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Proceedings of the National Academy of Sciences*. 103:8888-8893.

Doke, N., 1997. The oxidative burst: roles in signal transduction and plant stress. *Cold Spring Harbor Monograph Archive*. 34:785-813.

El Oirdi, M., El Rahman, T.A., Rigano, L., El Hadrami, A., Rodriguez, M.C., Daayf, F., Vojnov, A. and Bouarab, K., 2011. *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *The Plant Cell*. 23:2405-2421.

Fan, L.M., Zhao, Z. and Assmann, S.M., 2004. Guard cells: a dynamic signaling model. *Current opinion in plant biology*. 7:537-546.

Fernie, A.R., Carrari, F. and Sweetlove, L.J., 2004. Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Current opinion in plant biology.* 7:254-261.

Feys, B.J., Benedetti, C.E., Penfold, C.N. and Turner, J.G., 1994. Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *The Plant Cell*. 6:751-759.

Finkemeier, I., König, A.C., Heard, W., Nunes-Nesi, A., Pham, P.A., Leister, D., Fernie, A.R. and Sweetlove, L.J., 2013. Transcriptomic analysis of the role of carboxylic acids in metabolite signaling in Arabidopsis leaves. *Plant physiology*. 162:239-253.

Foyer, C.H. and Halliwell, B., 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta*. 133:21-25.

Fu, Z.Q. and Dong, X., 2013. Systemic acquired resistance: turning local infection into global defense. *Annual review of plant biology*. 64:839-863.

Galina, A., Reis, M., Albuquerque, M.C., Puyou, A.G., Puyou, M.T.G. and De Meis, L., 1995. Different properties of the mitochondrial and cytosolic hexokinases in maize roots. *Biochemical journal*. 309:105-112.

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M. and Toulmin, C., 2010. Food security: the challenge of feeding 9 billion people. *Science.* 327:812-818.

Goel, A.K., Lundberg, D., Torres, M.A., Matthews, R., Akimoto-Tomiyama, C., Farmer, L., Dangl, J.L. and Grant, S.R., 2008. The *Pseudomonas syringae* type III effector HopAM1 enhances virulence on water-stressed plants. *Molecular plant-microbe interactions*, 21:361-370.

Göhre, V. and Robatzek, S., 2008. Breaking the barriers: microbial effector molecules subvert plant immunity. *Annual Review Phytopathology.* 46:189-215.

Gomez, M.A., Alisaraie, L., Shio, M.T., Berghuis, A.M., Lebrun, C., Gautier-Luneau, I. and Olivier, M., 2010. Protein tyrosine phosphatases are regulated by mononuclear iron dicitrate. *Journal of Biological Chemistry*. 285:24620-24628.

Gómez-Gómez, L. and Boller, T., 2000. FLS2: An LRR receptor–like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular cell*. 5:1003-1011.

Grant, M. and Lamb, C., 2006. Systemic immunity. *Current opinion in plant biology*. 9:414-420.

Guerinot, M. L., 1995. Microbial iron transport. *Annual Reviews Microbiology*. 48:743–772

Haapalainen, M., van Gestel, K., Pirhonen, M. and Taira, S., 2009. Soluble plant cell signals induce the expression of the type III secretion system of *Pseudomonas syringae* and upregulate the production of pilus protein HrpA. *Molecular plant-microbe interactions*. 22:282-290.

Hewitson, K.S., Liénard, B.M., McDonough, M.A., Clifton, I.J., Butler, D., Soares, A.S., Oldham, N.J., McNeill, L.A. and Schofield, C.J., 2007. Structural and mechanistic studies on the inhibition of the hypoxia-inducible transcription factor hydroxylases by tricarboxylic acid cycle intermediates. *Journal of Biological Chemistry*. 282:3293-3301.

Higuchi, M., Pischke, M.S., Mähönen, A.P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S. and Helariutta, Y., 2004. In planta functions of the Arabidopsis cytokinin receptor family. *Proceedings of the National Academy of Sciences of the United States of America*. 101:8821-8826.

Hwang, I., Chen, H.C. and Sheen, J., 2002. Two-component signal transduction pathways in Arabidopsis. *Plant Physiology*. 129:500-515.

Ichihara, A., Shiraishi, K., Sato, H., Sakamura, S., Nishiyama, K., Sakai, R., Furusaki, A. and Matsumoto, T., 1977. The structure of coronatine. *Journal of the American Chemical Society*. 99:636-637.

Ingle, R.A., Carstens, M. and Denby, K.J., 2006. PAMP recognition and the plant–pathogen arms race. *Bioessays*. 28:880-889.

Jang, J.C. and Sheen, J., 1994. Sugar sensing in higher plants. *The Plant Cell*. 6:1665-1679.

Jang, J.C., León, P., Zhou, L. and Sheen, J., 1997. Hexokinase as a sugar sensor in higher plants. *The Plant Cell*. 9:5-19.

Janzen, D.J., Allen, L.J., MacGregor, K.B. and Bown, A.W., 2001. Cytosolic acidification and γ-aminobutyric acid synthesis during the oxidative burst in isolated *Asparagus sprengeri* mesophyll cells. *Canadian Journal of Botany.* 79:438-443.

Jelenska, J., Van Hal, J.A. and Greenberg, J.T., 2010. *Pseudomonas syringae* hijacks plant stress chaperone machinery for virulence. *Proceedings of the National Academy of Sciences*. 107:13177-13182.

Jones, J.D. and Dangl, J.L., 2006. The plant immune system. *Nature.* 444:323-329.

Jones, D.A. and Takemoto, D., 2004. Plant innate immunity–direct and indirect recognition of general and specific pathogen-associated molecules. *Current opinion in immunology*. 16:48-62.

Jones, A.M. and Wildermuth, M.C., 2011. The phytopathogen *Pseudomonas syringae pv. tomato DC3000* has three high-affinity iron-scavenging systems functional under iron limitation conditions but dispensable for pathogenesis. *Journal of bacteriology*. 193:2767-2775.

Journot-Catalino, N., Somssich, I.E., Roby, D. and Kroj, T., 2006. The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in *Arabidopsis thaliana*. *The Plant Cell*. 18:3289-3302.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E. and Shibuya, N., 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proceedings of the National Academy of Sciences*. 103:11086-11091.

Kaspar, S. and Bott, M., 2002. The sensor kinase CitA (DpiB) of *Escherichia coli* functions as a high-affinity citrate receptor. *Archives of microbiology*. 177:313-321.

Katagiri, F., Thilmony, R. and He, S.Y., 2002. The *Arabidopsis thaliana*-Pseudomonas syringae interaction. *The Arabidopsis Book*. e0039.

Kemmerling, B., Schwedt, A., Rodriguez, P., Mazzotta, S., Frank, M., Qamar, S.A., Mengiste, T., Betsuyaku, S., Parker, J.E., Müssig, C. and Thomma, B.P., 2007. The BRI1-associated kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control. *Current Biology.* 17:1116-1122.

Kim, M., Lim, J.H., Ahn, C.S., Park, K., Kim, G.T., Kim, W.T. and Pai, H.S., 2006. Mitochondria-associated hexokinases play a role in the control of programmed cell death in *Nicotiana benthamiana*. *The Plant Cell*. 18:2341-2355.

Lamb, C. and Dixon, R.A., 1997. The oxidative burst in plant disease resistance. *Annual review of plant biology*. 48:251-275.

Levine, A., Tenhaken, R., Dixon, R. and Lamb, C., 1994. H 2 O 2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell*. 79:583-593.

Li, X.Z., Starratt, A.N. and Cuppels, D.A., 1998. Identification of tomato leaf factors that activate toxin gene expression in *Pseudomonas syringae pv. tomato DC3000*. *Phytopathology*. 88:1094-1100.

Liu, J., Elmore, J.M., Lin, Z.J.D. and Coaker, G., 2011. A receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor. *Cell host & microbe*. 9:137-146.

Macho, A.P. and Zipfel, C., 2015. Targeting of plant pattern recognition receptor-triggered immunity by bacterial type-III secretion system effectors. *Current opinion in microbiology*. 23:14-22.

Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R. and Dangl, J.L., 2003. Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell*. 112:379-389.

Mansfield, J.W., 2009. From bacterial avirulence genes to effector functions via the hrp delivery system: an overview of 25 years of progress in our understanding of plant innate immunity. *Molecular plant pathology*. 10:721-734.

Marshall, B., Stintzi, A., Gilmour, C., Meyer, J.M. and Poole, K., 2009. Citrate-mediated iron uptake in *Pseudomonas aeruginosa*: involvement of the citrate-inducible FecA receptor and the FeoB ferrous iron transporter. *Microbiology*. 155:305-315.

Mathews, D.E. and Durbin, R.D., 1990. Tagetitoxin inhibits RNA synthesis directed by RNA polymerases from chloroplasts and Escherichia coli.*Journal of Biological Chemistry*. 265:493-498.

McCammon, M.T., Epstein, C.B., Przybyla-Zawislak, B., McAlister-Henn, L. and Butow, R.A., 2003. Global transcription analysis of Krebs tricarboxylic acid cycle mutants reveals an alternating pattern of gene expression and effects on hypoxic and oxidative genes. *Molecular biology of the cell*. 14:958-972.

McCann, H.C. and Guttman, D.S., 2008. Evolution of the type III secretion system and its effectors in plant–microbe interactions. *New Phytologist.* 177:33-47.

Melotto, M., Underwood, W., Koczan, J., Nomura, K. and He, S.Y., 2006. Plant stomata function in innate immunity against bacterial invasion. *Cell*. 126:969-980.

Meng, X. and Zhang, S., 2013. MAPK cascades in plant disease resistance signaling. *Annual Review of Phytopathology*. 51:245-266.

Meyer, S., De Angeli, A., Fernie, A.R. and Martinoia, E., 2010. Intra-and extra-cellular excretion of carboxylates. *Trends in plant science*. 15:40-47.

Miethke, M., Bisseret, P., Beckering, C.L., Vignard, D., Eustache, J. and Marahiel, M.A., 2006. Inhibition of aryl acid adenylation domains involved in bacterial siderophore synthesis. *FEBS Journal*. 273:409-419.

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W.H., Liu, Y.X., Hwang, I., Jones, T. and Sheen, J., 2003. Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal signaling. *Science*. 300:332-336.

Mur, L.A., Kenton, P., Atzorn, R., Miersch, O. and Wasternack, C., 2006. The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiology*. 140:249-262.

O'Donnell, P.J., Schmelz, E., Block, A., Miersch, O., Wasternack, C., Jones, J.B. and Klee, H.J., 2003. Multiple hormones act sequentially to mediate a susceptible tomato pathogen defense response. *Plant physiology*. 133:1181-1189.

Oerke, E.C., 2006. Crop losses to pests. *The Journal of Agricultural Science*. 144:31-43.

Olea, F., Pérez-García, A., Cantón, F.R., Rivera, M.E., Cañas, R., Ávila, C., Cazorla, F.M., Cánovas, F.M. and de Vicente, A., 2004. Up-regulation and localization of asparagine synthetase in tomato leaves infected by the bacterial pathogen *Pseudomonas syringae*. *Plant and cell physiology*. 45:770-780.

Østergaard, L. and Yanofsky, M.F., 2004. Establishing gene function by mutagenesis in *Arabidopsis thaliana*. *The Plant Journal*. 39:682-696.

Payne, S.M. and Neilands, I.B., 1988. Iron and virulence in the family Enterobacteriaceae. *CRC Critical reviews in microbiology*. 16:81-111.

Price, A.H., Taylor, A., Ripley, S.J., Griffiths, A., Trewavas, A.J. and Knight, M.R., 1994. Oxidative signals in tobacco increase cytosolic calcium. *The Plant Cell*. 6:1301-1310.

Rahme, L.G., Mindrinos, M.N. and Panopoulos, N.J., 1992. Plant and environmental sensory signals control the expression of hrp genes in *Pseudomonas syringae pv. phaseolicola*. *Journal of bacteriology*. 174:3499-3507.

Rate, D.N., Cuenca, J.V., Bowman, G.R., Guttman, D.S. and Greenberg, J.T., 1999. The gain-of-function Arabidopsis acd6 mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. *The Plant Cell*. 11:1695-1708.

Rico, A. and Preston, G.M., 2008. *Pseudomonas syringae pv. tomato DC3000* uses constitutive and apoplast-induced nutrient assimilation pathways to catabolize nutrients that are abundant in the tomato apoplast. *Molecular plant-microbe interactions*. 21:269-282.

Rico, A., McCraw, S.L. and Preston, G.M., 2011. The metabolic interface between *Pseudomonas syringae* and plant cells. *Current opinion in microbiology*. 14:31-38.

Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E.L., Kalkkinen, N., Romantschuk, M. and He, S.Y., 1997. Hrp pilus: an hrp-dependent bacterial surface appendage produced by *Pseudomonas syringae pv. tomato DC3000.Proceedings of the National Academy of Sciences*. 94:3459-3464.

Roitsch, T., 1999. Source-sink regulation by sugar and stress. *Current opinion in plant biology*. 2:198-206.

Roschzttardtz, H., Séguéla-Arnaud, M., Briat, J.F., Vert, G. and Curie, C., 2011. The FRD3 citrate effluxer promotes iron nutrition between symplastically disconnected tissues throughout Arabidopsis development. *The Plant Cell*. 23:2725-2737.

Salmeron, J.M. and Staskawicz, B.J., 1993. Molecular characterization and hrp dependence of the avirulence gene avrPro from *Pseudomonas syringae pv. tomato*. *Molecular and General Genetics*. 239:6-16.

Schneider, D.S., 2002. Plant immunity and film noir: what gumshoe detectives can teach us about plant-pathogen interactions. *Cell*. 109:537-540.

Scholl, R.L., May, S.T. and Ware, D.H., 2000. Seed and molecular resources for Arabidopsis. *Plant physiology*. 124:1477-1480.

Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M. and Waner, D., 2001. Guard cell signal transduction. *Annual review of plant biology*. 52:627-658.

Shelp, B.J., Bown, A.W. and McLean, M.D., 1999. Metabolism and functions of gamma-aminobutyric acid. *Trends in plant science*. 4:446-452.

Slabas, A.R., Ndimba, B., Simon, W.J. and Chivasa, S., 2004. Proteomic analysis of the Arabidopsis cell wall reveals unexpected proteins with new cellular locations. *Biochemical Society Transactions*. 32:524-528.

Steward, F.C., Thompson, J.F. and Dent, C.E., 1949. γ-Aminobutyric acid: a constituent of the potato tuber. *Science*. *110*:439-440.

Tang, X., Xiao, Y. and Zhou, J.M., 2006. Regulation of the type III secretion system in phytopathogenic bacteria. *Molecular plant-microbe interactions*. 19:1159-1166.

Thibaud, M.C., Gineste, S., Nussaume, L. and Robaglia, C., 2004. Sucrose increases pathogenesis-related PR-2 gene expression in *Arabidopsis thaliana* through an SA-dependent but NPR1-independent signaling pathway. *Plant Physiology and Biochemistry*. 42:81-88.

Truman, W., Zabala, M.T. and Grant, M., 2006. Type III effectors orchestrate a complex interplay between transcriptional networks to modify basal defence responses during pathogenesis and resistance. *The Plant Journal*. 46:14-33.

Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T. and Shinozaki, K., 1999. A transmembrane hybrid-type histidine kinase in Arabidopsis functions as an osmosensor. *The Plant Cell.* 11:1743-1754.

Van Wees, S.C., De Swart, E.A., Van Pelt, J.A., Van Loon, L.C. and Pieterse, C.M., 2000. Enhancement of induced disease resistance by simultaneous activation of salicylate-and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*. 97:8711-8716.

Vanacker, H., Carver, T.L. and Foyer, C.H., 1998. Pathogen-induced changes in the antioxidant status of the apoplast in barley leaves. *Plant Physiology*. 117:1103-1114.

Veronese, P., Nakagami, H., Bluhm, B., AbuQamar, S., Chen, X., Salmeron, J., Dietrich, R.A., Hirt, H. and Mengiste, T., 2006. The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in Arabidopsis resistance to necrotrophic and biotrophic pathogens. *The Plant Cell*. 18:257-273.

Wang, L., Mitra, R.M., Hasselmann, K.D., Sato, M., Lenarz-Wyatt, L., Cohen, J.D., Katagiri, F. and Glazebrook, J., 2008. The genetic network controlling the Arabidopsis transcriptional response to *Pseudomonas syringae pv. maculicola*: roles of major regulators and the phytotoxin coronatine. *Molecular plant-microbe interactions*. 21:1408-1420.

Ward, J.L., Forcat, S., Beckmann, M., Bennett, M., Miller, S.J., Baker, J.M., Hawkins, N.D., Vermeer, C.P., Lu, C., Lin, W. and Truman, W.M., 2010. The metabolic transition during disease following infection of *Arabidopsis thaliana* by *Pseudomonas syringae pv. tomato*. *The Plant Journal*. 63:443-457.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V. and Provart, N.J., 2007. An “Electronic Fluorescent Pictograph” browser for exploring and analyzing large-scale biological data sets. *PloS one*. 2:e718.

Wolffram, S., Zimmermann, W. and Scharrer, E., 1993. Transport of tricarballylate by intestinal brush-border membrane vesicles from steers.*Experimental physiology*. 78:473-473.

Xiang, T., Zong, N., Zou, Y., Wu, Y., Zhang, J., Xing, W., Li, Y., Tang, X., Zhu, L., Chai, J. and Zhou, J.M., 2008. *Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. *Current Biology*. 18:74-80.

Zeidler, D., Zähringer, U., Gerber, I., Dubery, I., Hartung, T., Bors, W., Hutzler, P. and Durner, J., 2004. Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proceedings of the National Academy of Sciences of the United States of America*. 101:15811-15816.

Chapter 6: Supplementary Data

Table S1: **Differential expression of known defence genes post citrate treatment.**

Each term is represented by Atg number, name (if given), molecular (functions in) function and biological process (involved in). The table is divided vertically into citrate down regulated and citrate upregulated defence genes. All data was obtained from Finkemeier et al (2013).

Number	Name	Molecular function	Biological process
CITRATE DOWN REGULATED DEFENCE (GO) GENES			
at1g04370	Ethylene-responsive element binding factor 14 (ATERF14)	Transcription factor activity, DNA binding	Oxidation reduction, defence response to bacterium
at1g10920	Locus Orchestrating VIctorin effects 1 (LOV1)	ATP binding	Stomatal complex patterning, floral organ abscission, defence response, incompatible interaction, stomatal complex development
at1g12820	Auxin signalling F-Box 3 (AFB3)	Auxin binding, ubiquitin-protein ligase activity	Oxygen and reactive oxygen species metabolic process, response to heat, negative regulation of programmed cell death, defence response
at1g19570	Dehydroascorbate Reductase (DHAR1)	Glutathione dehydrogenase (ascorbate) activity	Unknown
at1g19610	Plant Defensin 1.4 (PDF1.4)	Unknown	Defence response
at1g20020	Leaf FNR 2 (ATLFNR2)	Oxidoreductase activity, poly(U) binding, NADPH dehydrogenase activity	Defence response
at1g22900		Unknown	Protein folding, response to cadmium ion, signal transduction
at1g31580	ECS1	Unknown	Response to cold, defence response to bacterium
at1g47540	Trypsin inhibitor	Ion channel inhibitor activity	Response to cold, defence response to bacterium
at1g52740	Histone H2A PROTEIN 9 (HTA9)	DNA binding	Defence response to bacterium
at1g55010	Plant Defensin 1.5 (PDF1.5)	Unknown	Defence response to bacterium, incompatible interaction, cell death, response to salicylic acid stimulus
at1g70830	MLP-Like Protein 28 (MLP28)	Unknown	Stamen development, pollen maturation, response to molecule of bacterial origin
at1g72940	Disease resistance	Transmembrane receptor	Oxidation reduction,

	protein (TIR-NBS class)	activity	defence response to bacterium
at1g75040	Pathogenesis-related 5 (PR5)	Unknown	Protein folding, response to cadmium ion, signal transduction
at1g75830	Low-molecular-weight Cysteine-rich 67 (LCR67)	Unknown	Signal transduction, innate immune response
at2g02100	Low-molecular-weight Cysteine-rich 69 (LCR69)	Peptidase inhibitor activity	Stomatal complex patterning, floral organ abscission, defence response, incompatible interaction, stomatal complex development
at2g02120	Plant Defensin 2.1 (PDF2.1)	Peptidase inhibitor activity	Defence response to bacterium
at2g02130	Low-molecular-weight Cysteine-rich 68 (LCR68)	Peptidase inhibitor activity	Oxygen and reactive oxygen species metabolic process, response to heat, negative regulation of programmed cell death, defence response
at2g14560	Late Upregulated in Response to *Hyaloperonospora Parasitica* (LURP1)	Defence response to fungus, response to salicylic acid stimulus	Defence response to bacterium, regulation of flower development
at2g20142	Transmembrane receptor	Transmembrane receptor activity	Defence response
at2g26010	Plant Defensin 1.3 (PDF1.3)		Stomatal complex patterning, floral organ abscission, defence response, incompatible interaction, stomatal complex development
at2g26020	Plant Defensin 1.2b (PDF1.2b)	Unknown	Oxidation reduction, defence response to bacterium
at2g32680	Receptor Like Protein 23 (AtRLP23)	Protein binding, kinase activity	Ethylene mediated signaling pathway, response to chitin, regulation of transcription, DNA-dependent
at2g40750	WRKY54	Transcription factor activity	Defence response
at2g43520	Trypsin Inhibitor protein 2 (ATTI2)	Serine-type endopeptidase inhibitor activity	Signal transduction, defence response, innate immune response
at2g43530	Trypsin inhibitor	Ion channel inhibitor activity	Defence response
at2g43535	Trypsin inhibitor	Ion channel inhibitor activity	Signal transduction, innate immune response
at2g43550	Trypsin inhibitor	Ion channel inhibitor activity	Defence response
at3g01500	CARBONIC ANHYDRASE 1 (CA1)	Carbonate dehydratase activity, zinc ion binding	Defence response
at3g11630	2-cys peroxiredoxin, chloroplast (BAS1);	Peroxiredoxin activity, Antioxidant activity	Signal transduction, innate immune response
at3g21220	Arabidopsis Thaliana Mitogen-Activated Protein Kinase Kinase 5	MAP kinase kinase activity, kinase activity	Defence response to bacterium, regulation of flower development

	(ATMKK5)		
at3g23120	Receptor Like Protein 38 (AtRLP38)	Protein binding, kinase activity	Response to biotic stimulus, defence response
at3g26520	Tonoplast Intrinsic Protein 2 (TIP2)	Water channel activity	Stamen development, pollen maturation, response to molecule of bacterial origin
at3g62030	Rotamase CyP 4 (ROC4)	Peptidyl-prolyl cis-trans isomerase activity	9 processes
at4g14400	Accelerated Cell Death 6 (ACD6)	Protein binding	Ethylene mediated signaling pathway, response to chitin, regulation of transcription, DNA-dependent
at4g17490	Ethylene Responsive element binding Factor 6 (ATERF6)	Transcription factor activity, DNA binding	Signal transduction, defence response, innate immune response
at4g32260	ATP synthase family	Hydrogen ion transmembrane transporter activity	Defence response to fungus, response to molecule of fungal origin, defence response
at4g38740	Rotamase CYP 1 (ROC1)	Peptidyl-prolyl cis-trans isomerase activity	Oxidation reduction, defence response to bacterium
at5g20900	Jasmonate-Zim-domain protein 12 (JAZ12)	Unknown	Unknown
at5g42500	Disease resistance-responsive family protein	Unknown	Defence response
at5g44420	Plant Defensin 1.2 (PDF1.2)	6 processes	Oxidation reduction, defence response to bacterium
at5g44430	Plant Defensin 1.2c (PDF1.2c)		Oxygen and reactive oxygen species metabolic process, response to heat, negative regulation of programmed cell death, defence response
at5g47910	Respiratory Burst Oxidase Homologue D (RBOHD)	NAD(P)H oxidase activity	Unknown
at5g58090	Glycosyl hydrolase family 17 protein	Cation binding, hydrolase activity, hydrolyzing O-glycosyl compounds, catalytic activity	Defence response to fungus, response to molecule of fungal origin, defence response
at5g66190	Ferredoxin-NADP(+)-oxidoreductase 1 (FNR1)	Electron transporter, oxidoreductase activity, poly(U) binding, NADPH dehydrogenase activity, transferring electrons within the cyclic and noncyclic electron transport pathway of photosynthesis activity	Defence response to bacterium, incompatible interaction, cell death, response to salicylic acid stimulus
CITRATE UP REGULATED DEFENCE (GO) GENES			

at1g02920	Glutathione S-Transferase (GST11)		
at4g10780	Disease resistance protein (CC-NBS-LRR class)	Protein binding, nucleoside-triphosphatase activity, nucleotide binding, ATP binding	N-terminal protein myristoylation, defence response, apoptosis
at4g26850	Vitamin c defective 2 (VTC2)	8 functions	Response to jasmonic acid stimulus, L-ascorbic acid biosynthetic process, defence response to bacterium, response to heat, callose deposition in cell wall during defence response
at4g36690	Splicing factor U2af large subunit A (ATU2AF65A)	RNA binding, nucleotide binding, nucleic acid binding	Nuclear mRNA splicing, via spliceosome, defence response to bacterium
at4g36130	60S ribosomal protein L8 (RPL8C)	Structural constituent of ribosome	Translation
at3g05200	ATL6	Protein binding, zinc ion binding	Response to chitin
at1g66340	Ethylene Response 1 (ETR1)	Ethylene binding, two-component response regulator activity, protein histidine kinase activity	16 processes
at4g33430	BRI1-Associated receptor Kinase (BAK1)	Protein binding, protein serine/threonine kinase activity, protein heterodimerization activity, kinase activity	6 processes
at4g36140	Disease resistance protein (TIR-NBS-LRR class)	Protein binding, transmembrane receptor activity, nucleoside-triphosphatase activity, nucleotide binding, ATP binding	Signal transduction, defence response, apoptosis, innate immune response
at5g04720	ADR1-like 2 (ADR1-L2)	Protein binding, nucleoside-triphosphatase activity, nucleotide binding, ATP binding	Defence response, apoptosis
at5g44910	Toll-Interleukin-Resistance (TIR)	Transmembrane receptor activity	Signal transduction, defence response, innate immune response
at5g44870	Disease resistance protein (TIR-NBS-LRR class)	Protein binding, transmembrane receptor activity, ATP binding	Signal transduction, defence response, apoptosis, innate immune response
at5g15090	Voltage Dependent Anion Channel 3	Voltage-gated anion	Defence response to

	(VDAC3)	channel activity	bacterium, response to bacterium, anion transport
at2g27500	Glycosyl hydrolase family 17 protein	Cation binding, hydrolase activity, hydrolyzing O-glycosyl compounds, catalytic activity	Carbohydrate metabolic process
at5g09650	*Arabidopsis thaliana* pyrophosphorylase 6 (AtPPa6)	Inorganic diphosphatase activity, pyrophosphatase activity	7 processes
at4g16760	ACYL-COA Oxidase 1 (ACX1)	Acyl-CoA oxidase activity	Response to cadmium ion, fatty acid beta-oxidation, long-chain fatty acid metabolic process, response to wounding
at1g10170	*Arabidopsis Thaliana* NF-X-Like 1 (ATNFXL1)	Protein binding, transcription factor activity, zinc ion binding	6 processes
at1g58170	Disease resistance-responsive protein-related / dirigent protein-related	Unknown	N-terminal protein myristoylation, lignan biosynthetic process, defence response
at1g72910	Disease resistance protein (TIR-NBS class)	Transmembrane receptor activity	Signal transduction, defence response, innate immune response
at3g15020	Malate dehydrogenase (NAD)	6 functions	Defence response to bacterium
at4g16990	Resistance to *Leptosphaeria Maculans* 3 (RLM3)	Transmembrane receptor activity, ATP binding	Defence response to fungus, incompatible interaction, jasmonic acid and ethylene-dependent systemic resistance, callose deposition during defence response, defence response
at1g72920	Disease resistance protein (TIR-NBS class)	Transmembrane receptor activity	Response to wounding, defence response
at2g39200	Mildew resistance Locus O 12 (MLO12)	Calmodulin binding	Defence response to fungus, incompatible interaction, response to cyclopentenone, cell death, defence response
at3g26810	Auxin Signaling F-Box 2 (AFB2)	Auxin binding, ubiquitin-protein ligase activity	Stamen development, pollen maturation, response to molecule of bacterial origin
at4g12470	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	Lipid binding	Defence response to fungus, lipid transport

at5g44700	GASSHO 2 (GSO2)	Protein binding, protein serine/threonine kinase activity, protein tyrosine kinase activity, protein kinase activity, ATP binding	Embryonic development, transmembrane receptor protein tyrosine kinase signaling pathway, protein amino acid phosphorylation, embryo sac development, epidermis development
at5g51070	Early Responsive to Dehydration 1 (ERD1)	Protein binding, nucleoside-triphosphatase activity, ATPase activity, nucleotide binding, ATP binding	Response to water deprivation
at5g05170	Constitutive Expression of VSP 1 (CEV1)	Cellulose synthase activity, transferase activity, transferring glycosyl groups	Primary cell wall biogenesis, cellulose biosynthetic process, defence response
at5g45050	Tolerance to Tobacco Ringspot virus 1 (TTR1)	Protein binding, transcription factor activity	Response to virus, plant-type hypersensitive response
at5g64440	*Arabidopsis thaliana* fatty acid amide hydrolase (AtFAAH)	N-(long-chain-acyl)ethanolamine deacylase activity, amidase activity	Defence response to bacterium
at4g33300	ADR1-like 1 (ADR1-L1)	Protein binding, ATP binding	Defence response, apoptosis
at4g31550	WRKY11	Transcription factor activity, calmodulin binding	Defence response to bacterium, response to chitin, regulation of transcription, DNA-dependent
at4g12480	pEARLI 1	Lipid binding	Defence response to fungus, lipid transport
at2g47730	*Arabidopsis thaliana* Glutathione S-Transferase phi 8 (ATGSTF8)	glutathione transferase activity, glutathione binding	7 processes
at2g39660	Botrytis-Induced Kinase1 (BIK1)	Kinase activity	Defence response to fungus, protein amino acid autophosphorylation, N-terminal protein myristoylation, response to fungus
at4g31500	Cytochrome P450 monooxygenase 83B1 (CYP83B1)	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NADH or NADPH as one donor, and incorporation of one atom of oxygen, oxygen binding	8 processes
at4g39950	CYP79B2	Electron carrier activity,	6 processes

		monooxygenase activity, iron ion binding, oxygen binding, heme binding	
at4g03550	Glucan Synthase-Like 5 (ATGSL05)	Transferase activity, transferring glycosyl groups, 1,3-beta-glucan synthase activity	9 processes
at1g61560	Mildew Resistance Locus O 6 (MLO6)	Calmodulin binding	Defence response to fungus, incompatible interaction, cell death, defence response
at2g38310	Unknown protein	Unknown	
at4g12490	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	Lipid binding	Defence response to fungus, lipid transport
at5g56030	Heat Shock Protein 81-2 (HSP81-2)	ATP binding	9 processes
at1g09665	Toll-Interleukin-Resistance (TIR) domain-containing protein	Transmembrane receptor activity	Defence response signaling pathway, resistance gene-dependent, signal transduction, defence response, innate immune response
at4g23100	Glutamate-Cysteine Ligase (GSH1)	Glutamate-cysteine ligase activity	13 processes
at3g54420	ATEP3	Chitinase activity	Somatic embryogenesis, plant-type hypersensitive response
at4g11280	1-Aminocyclopropane-1-Carboxylic Acid (ACC) Synthase 6 (ACS6)	1-aminocyclopropane-1-carboxylate synthase activity	8 processes
at2g30860	Glutathione S-Transferase phi 9 (ATGSTF9)	Glutathione transferase activity, glutathione peroxidase activity	Defence response to bacterium, toxin catabolic process, defence response
at2g19190	FLG22-Induced Receptor-like Kinase 1 (FRK1)	Kinase activity	Defence response to bacterium
at3g54640	Tryptophan Synthase Alpha chain (TSA1)	Tryptophan synthase activity	Tryptophan biosynthetic process, defence response to bacterium, callose deposition in cell wall during defence response
at5g06860	Polygalacturonase Inhibiting Protein 1 (PGIP1)	Protein binding	Signal transduction, defence response
at5g06870	Polygalacturonase Inhibiting Protein 2	Protein binding	Signal transduction,

	(PGIP2)		response to salt stress, defence response
at5g39580	Peroxidase	Electron carrier activity, peroxidase activity, heme binding	Defence response to fungus, N-terminal protein myristoylation
at2g23150	Natural Resistance-Associated Macrophage Protein 3 (NRAMP3)	Manganese ion transmembrane transporter activity, inorganic anion transmembrane transporter activity, metal ion transmembrane transporter activity	9 processes

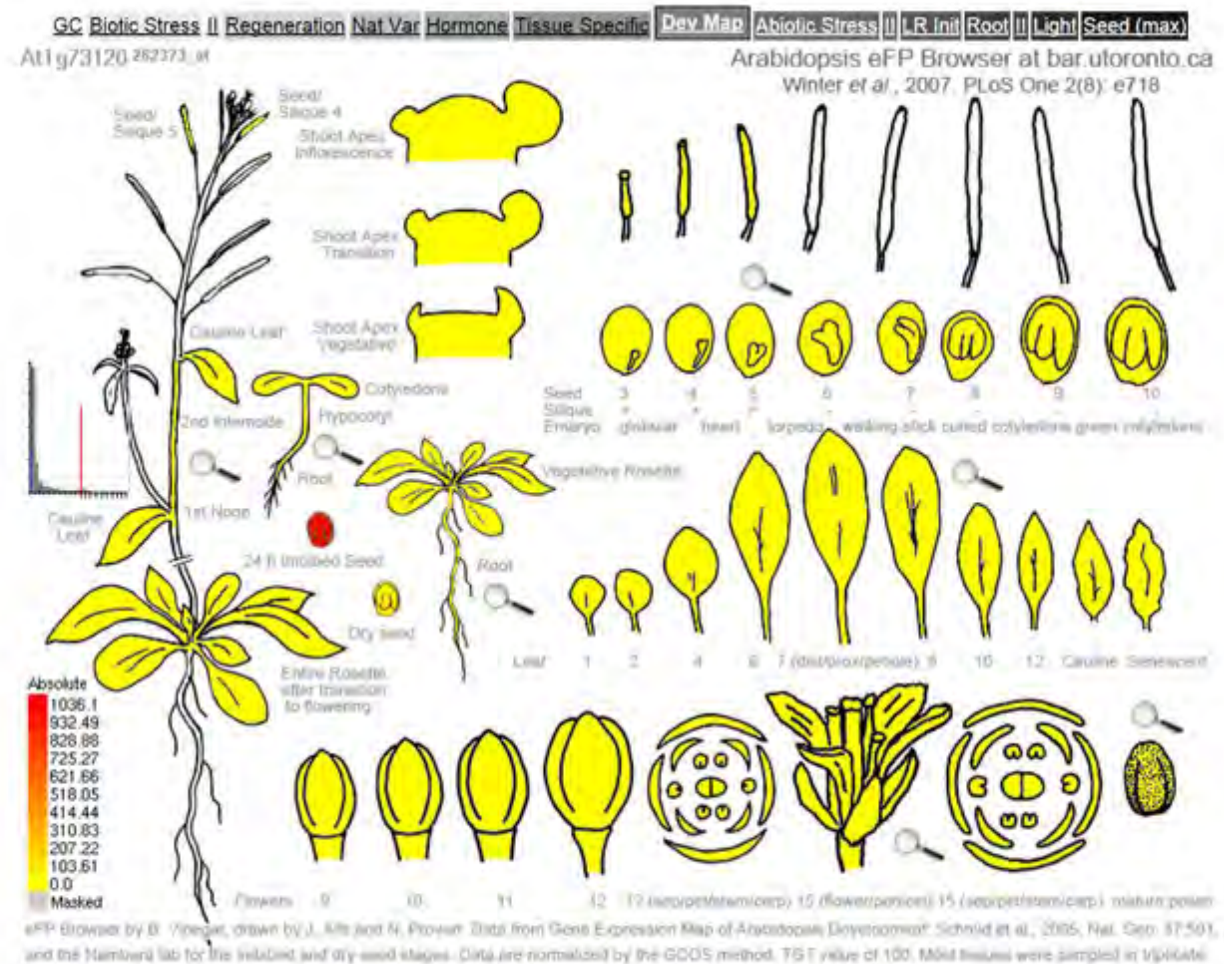

Figure S1: **Electronic fluorescent pictograph of gene expression in the Arabidopsis developmental map shows minimal *CI1* expression.**

CI1 expression was analysed in Arabidopsis developmental stages using the Arabidopsis eFP tool (http://bar.utoronto.ca). No *CI1* expression was observed in any of plant stages (yellow), with the exception of the 24 hour imbibed seed (red), which displayed high levels of expression.

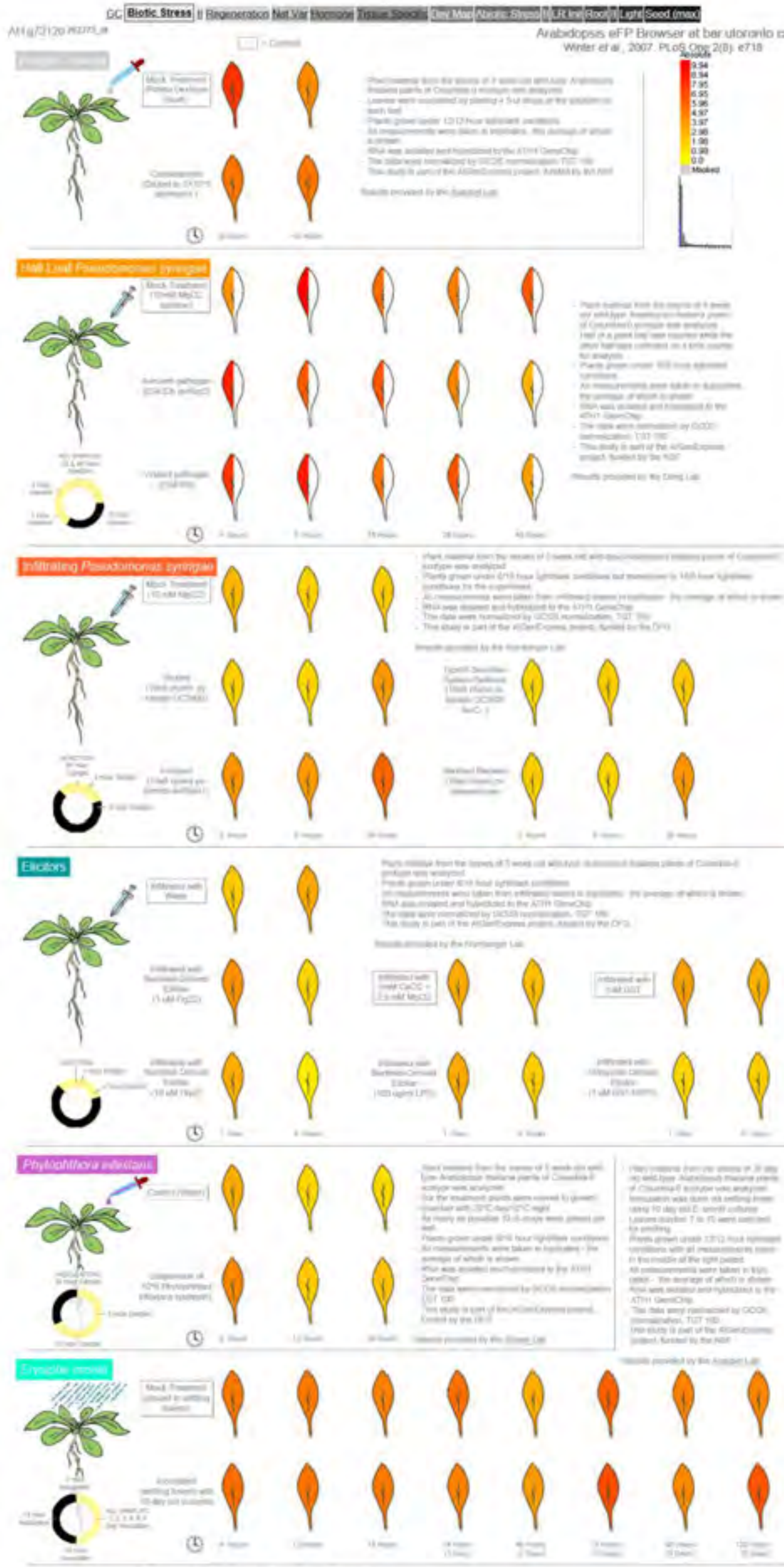

Figure S2: **Electronic fluorescent pictograph of gene expression during biotic stress in Arabidopsis shows low level *CI1* expression.**

The Arabidopsis eFP tool was used to analyse *CI1* expression in leaves exposed to biotic stress, including *Pst, Botrytic cinerea* and various pathogen elicitors. Low levels of *CI1* expression was observed in the *B. cinerea*, *Pst* half leaf and *Erysiphe orontii* treatments.