

DIR1 MOVEMENT DURING SAR & DEVELOPMENT OF AN ARR SCREEN

DIR1 MOVEMENT DURING LONG DISTANCE SIGNALING IN
SYSTEMIC ACQUIRED RESISTANCE
&
DEVELOPMENT OF A FLORESCENCE BASED SCREEN FOR
AGE-RELATED RESISTANCE MUTANTS

By

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Abstract

Systemic Acquired Resistance (SAR) is an induced resistance mechanism in which certain localized pathogen infections lead to broad resistance against future attacks in distant tissues. Resistance is transmitted from induced tissues by long distance signals that are perceived in systemic tissue eliciting defence to normally virulent pathogens. Previous research in the Cameron laboratory indicates that DIR1 may function as a long distance signal during SAR. *Agrobacterium* transient transformation was used to study DIR1 movement in the *dir1-1* SAR-defective mutant. Lower leaves of *dir1-1* were infiltrated with *Agrobacterium* expressing 35S:DIR1-EYFP, followed by inoculation with SAR-inducing *Pseudomonas syringae* pv. *tomato* (*Pst avrRpt2*). Distant leaves were challenged with virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) and bacterial levels were determined. The SAR defect was rescued in *dir1-1* plants expressing 35S:DIR1-EYFP only if SAR was induced. Petiole exudates were collected from these induced leaves and DIR1 was present, suggesting that DIR1 is capable of movement during SAR. Additionally, when cucumber exudates induced for SAR were infiltrated into *dir1-1* Arabidopsis, *dir1-1* became SAR-competent. Cucumber exudates contained a DIR1-like protein. Taken together, these data suggest that a DIR1-like protein exists in cucumber and functions like DIR1 in Arabidopsis during SAR.

Age-Related Resistance (ARR) has been observed in numerous plant species, often resulting in increased disease resistance as plants mature. A genetic screen to identify potential ARR mutants has been developed. The screen used a fluorescence strain of *Pst* (*Pst-GFPuv*) to quantify bacterial levels in infected leaves. A number of parameters were investigated to improve the screen.

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Abbreviations

ARR	Age-Related Resistance
<i>avr</i>	avirulence gene
BLAST	basic local alignment search tool
cDNA	complementary deoxyribonucleic acid
cfu	colony forming unit
Col-0	Columbia ecotype of Arabidopsis
dH ₂ O	distilled water
<i>dir1-1</i>	<i>defective in induced resistance 1-1</i>
DNA	deoxyribonucleic acid
dpg	days post germination
dpi	days post inoculation
HR	hypersensitive response
JA	jasmonic acid
KB	King's B media
MgCl ₂	magnesium chloride
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
<i>npr1-2</i>	<i>non-expressor of PR-1</i>
OD	optical density
PCR	polymerase chain reaction
PR	pathogenesis-related
<i>Pst</i>	<i>Pseudomonas syringae</i> pv. <i>tomato</i>
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
TAIR	The <i>Arabidopsis</i> Information Resource
Tris	Tris-(hydroxymethyl)aminomethane
UTR	untranslated region
Ws-0	Wassilewskija ecotype of Arabidopsis

Introduction

1.1 Plants and Disease

Plants are physically rooted to the ground, and can not relocate to escape unfavourable conditions such as the absence of nutrients, water and light (abiotic stresses) or pathogen attack from bacteria, fungi and viruses (biotic stresses) (Jones and Dangl, 2006). Plants lack a vertebrate-like circulatory system and antibodies to protect themselves against pathogen attack. Instead plants have evolved a number of rapid, sophisticated defense mechanisms including basal defense, R gene-mediated defense, Age-Related Resistance (ARR) and Systemic Acquired Resistance (SAR) (Staskawicz et al., 1995; Jones and Dangl, 2006).

1.2 Basal Defense

Basal defense can be described as a broad, non-specific defense where an entire plant species is resistant to a number of pathogens (Jones and Dangl, 2006). This defense strategy is a combination of constitutive and inducible defense mechanisms that are not induced by the presence of a specific pathogen, but instead by common pathogen molecules (Jones and Dangl, 2006). Pathogen-associated molecular patterns (PAMPs) (also called microbe-associated molecular patterns [MAMPs]) are extracellular microbial molecules or structures. PAMPs are not present in their plant host and therefore can be perceived as 'non-self' by host encoded PAMP-receptors (Jones and Dangl, 2006).

PAMPs are recognized by plant transmembrane pattern recognition receptors (PRRs), resulting in PAMP-triggered immunity (PTI) or basal defense (Jones and Dangl, 2006). A classic example of a PAMP is flagellin, the structural protein of the bacterial flagellum (Nurnberger et al., 2004) which is recognized by Arabidopsis PAMP-receptor Flagellin Sensitive 2 (FLS2). Recognition leads to activation of the basal resistance pathway that leads to production of secondary metabolites that are antimicrobial (such as phenolics and alkaloids), cell wall modifications that strengthen the cell wall such as the introduction of lignin, suberin, silica and hydroxyproline-rich glycoproteins and the production of active oxygen species (AOS) that may act in an antimicrobial manner (Jones and Dangl, 2006). Basal defense also includes expression of defense genes such as pathogenesis-related (PR) genes (Kim et al., 2008). These defense strategies work together to limit pathogen growth thus conferring a generalized disease resistance to the host (Jones and Dangl, 2006). However many pathogens are not slowed by these strategies and others have evolved a number of mechanisms to overcome the plants' basal defense strategy (Jones and Dangl, 2006). Some bacterial pathogens such as *Pseudomonas syringae* release effector molecules (also referred to as virulence factors) into the plant cells that interfere with basal resistance (Jones and Dangl, 2006). The pathogen effectors are delivered through a type III secretion system (T3SS), encoded by the *hypersensitive response and pathogenicity (hrp)* locus. The T3SS produces a pilus-like structure that allows bacterial proteins to cross the plant cell wall and membrane. Effectors contribute to the pathogen's

virulence by interfering with basal defense resulting in effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). Conservative estimates suggest that some pathogenic bacteria deliver 15 to 30 effectors per strain to their host (Jones and Dangl, 2006) whereas others suggest this number could be as high as 300 (Cui, Xiang and Zhou, 2009). Bacterial effectors are thought to contribute to virulence by mimicking plant cellular functions, inhibiting plant cell functions, promoting nutrient leakage, aiding in pathogen dispersal or directly suppressing components of basal defense (Jones and Dangl, 2006). Fungal and oomycete effectors are poorly understood, but are thought to act in the extracellular matrix or inside the plant cell (Jones and Dangl, 2006). The delivery mechanism and how these eukaryotic effectors contribute to pathogen virulence is not understood (Jones and Dangl, 2006). Effectors also contribute to disease resistance as plants have evolved the ability to recognize some of these effectors. Plants produce protein receptors that recognize specific effectors, if there is an effector-receptor match the effector is now referred to as an avirulence (avr) protein, the gene that encodes the receptor is called a Resistance gene (R gene) and the interaction of the two is R gene-mediated resistance (Jones and Dangl, 2006).

1.3 R gene-mediated resistance and the Hypersensitive Response

When a plant R gene product recognizes a pathogen avr gene product the plant host can activate a number of defense responses that result in limited pathogen growth and prevent disease. A successful R gene-avr interaction is a faster and stronger version of basal defense referred to as Effector Triggered Immunity (ETI) or R gene-mediated resistance. It is thought that a single R gene product can recognize a single avr gene product to trigger a common signaling pathway that leads to production and accumulation of Active Oxygen Species (AOS), known as the oxidative burst, rapid ion influxes, followed by salicylic acid (SA) accumulation, expression of Pathogenesis-related (PR) genes and hypersensitive response (HR)-associated cell death (Jones and Dangl, 2006). The HR is a form of localized cell death at and near the site of infection, it typically doesn't extend outside of the infected cells and is thought to limit pathogen growth in some situations but the mechanism by which this occurs is not clear (Kim et al., 2008). Arabidopsis ecotype Col-0 is predicted to have 149 R gene products based on sequence analysis for common structural features (Eckardi and Innes, 2003; Meyers et al., 2003). Many of these R genes encode a centrally located Nucleotide Binding site and a Leucine Rich Repeat region at the C-terminal. Nucleotide Binding Leucine Rich Repeat (NB-LRR) proteins can further be divided into two families based on the N-terminal sequence. One family contains a coiled-coil motif (CC-NB-LRR) and the other has a Toll-like motif (TIR-NB-LRR) (Kim et al., 2008). The mechanism of recognition of an avr product by a

one of these R gene receptors is poorly understood (Kim et al., 2008). There is some evidence that R gene receptors can interact directly with avr gene products (Jia et al., 2000) but many do not (Mackey et al., 2002) suggesting that R gene products may form complexes with other plant proteins and the avr product.

The pathogen avirulence gene products can be delivered through the T3SS into the cytosol or interaction of the avirulence gene product and the R receptor can occur in the intercellular space. An example of intercellular interactions is the presence of extracytoplasmic leucine-rich repeats (LRR) domains that interact with some fungal avirulence genes that are secreted to the intercellular space of the plant (Jones and Dangl, 2006). If the plant lacks the appropriate R protein or the pathogen lacks the avirulence proteins then R gene-mediated resistance will not occur as the plant will not recognize the pathogen (Jones and Dangl, 2006).

Plant-pathogen interactions that lead to basal defense and R gene-mediated resistance can be summarized in the 'zig-zag' model proposed by Jones and Dangl (2006) (Figure 1). This model illustrates the quantitative outputs of the plant immune response. The four stage model begins with plant receptors (PRRs) detecting pathogen molecules (PAMPs) to initiate basal defense. Successful pathogens deliver effectors into the plant cells that interfere with basal defense or otherwise enhance pathogen growth and reproduction, resulting in effector triggered susceptibility. Stage three occurs if a single pathogen effector is specifically recognized by a plant resistance receptor activating R

gene-mediated resistance. In the final stage pathogens evolve new effectors or gain new effectors through horizontal gene transfer. These new effectors may not be recognized by the plant's current array of receptors and disease will result (Jones and Dangl, 2006).

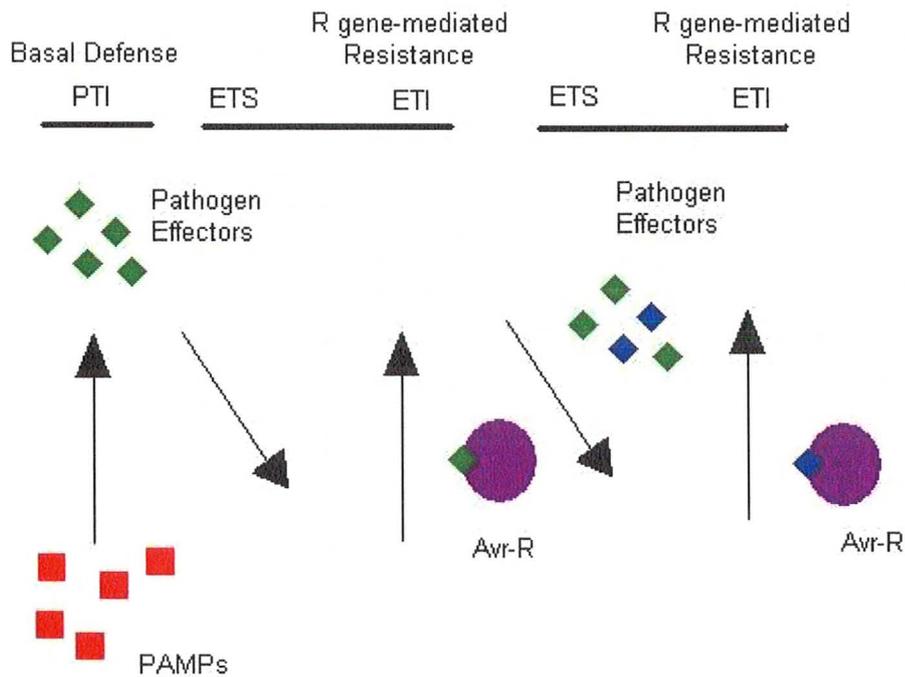


Figure 1: The 'zig-zag' model summarizes plant-pathogen interactions. In the first phase, plant receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs, red square) to trigger basal defense (also called PAMP-triggered immunity [PTI]). In the second phase, pathogens deliver effectors that interfere with basal defense, resulting in effector-triggered susceptibility (ETS). In phase three, a single effector (green diamond) is recognized by a plant R gene receptor (purple circle) activating R gene-mediated resistance (also called effector-triggered immunity [ETI]). R gene-mediated resistance is an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase four, pathogen isolates are selected that have lost the effector (green diamond). This pathogen may have new effectors (blue diamond) which are capable of suppressing basal defense. Selection then favours new plant resistance receptors that can recognize one of the newly acquired effectors, resulting once again in R gene-mediated resistance (Based on Dangl and Jones, 2006).

1.4 Age-Related Resistance

1.4.1 Definition of Age-Related Resistance and Examples

Age-Related Resistance (ARR) is a form of defense in which older plants display increased resistance to pathogens compared to young individuals of the same genotype (Kus et al., 2002). The relationship between plant age and susceptibility to pathogens is somewhat variable depending on the particular plant and pathogen involved. For example, as rice plants age susceptibility to *Xanthomonas campestris* pv *oryzae* decreases (Miller, 1983). Conversely some plants become more susceptible to particular pathogens as they age, for example, onion and *Alternaria porri* (Miller, 1983).

Numerous plant-pathogen pairings result in ARR (plant is less susceptible with age) in addition to the aforementioned rice and *Xanthomonas-campestris* pv *oryzae* example. Other plant-bacteria pairs include Arabidopsis-*Pseudomonas syringae* pv. *tomato* (Kus et al., 2002), pepper plants-*Phytophthora capsici* (Kim, Hwang, Park, 1989), as well as soybeans-*Phytophthora sojae*. Examples of plants capable of mounting ARR responses to viruses include Arabidopsis-cauliflower mosaic virus (Leisner et al., 1993) and tobacco-tobacco mosaic virus (Yalpani et al., 1993). ARR to oomycetes has been studied in the Arabidopsis-*Hyaloperonospora arabidopsidis* (formerly *H. parasitica*) (Rusterucci et al., 2005), tobacco-*Phytophthora parasitica* (Hugot et al 1999), bean-*Colletotrichum lindemuthianum* (Griffey and Leach, 1965), tomato-*Cladosporium fulvum* and wheat-*Puccinia graminis* f. sp. *tritici* (Sunderwirth and Roelfs, 1980).

1.4.2 ARR Pathway in Arabidopsis

Whether ARR affords broad-spectrum resistance to a diverse array of pathogens has not been fully addressed. However, mature tobacco plants become more resistant to *Peronospora tabacina* (Wyatt et al., 1991), *Phytophthora parasitica* (Hugot et al., 1999) and tobacco mosaic virus. Arabidopsis exhibits ARR to *Pseudomonas syringae* pv. *tomato* (*Pst*), pv. *maculicola* (*Psm*) and the oomycete *Hyaloperonospora arabidopsidis* (Rusterucci et al., 2005). During a typical ARR response in Arabidopsis to *Pst*, *Pst*-induced chlorotic disease symptoms are reduced and an HR-like response is not observed (Kus et al., 2002). Virulent *Pst* growth is reduced 10- to 100-fold and *H. arabidopsidis* conidiospore production is reduced five- to 50-fold (Kus et al., 2002; Rusterucci et al., 2005). The ARR response is effective to both oomycetes and bacteria suggesting that ARR is not a form of basal defense because the PAMPs present in *Pst* are likely different from the PAMPs found in *H. arabidopsidis*. Lack of HR suggests that ARR is not a form of R gene-mediated resistance. In a standard Arabidopsis-*Pst* ARR experiment, wild-type Col-0 plants display ARR at approximately six weeks post germination (wpg) under short day conditions (9 hours of light). Studies suggest the transition to flowering is associated with ARR competence (Kus et al., 2002, Rusterucci et al., 2005). Salicylic acid (SA) accumulation in the intercellular space is required for the Arabidopsis ARR response as demonstrated by the lack of ARR in SA-deficient mutants NahG, *sid1* and *sid2* (Kus et al., 2002). SA is a phenolic compound produced through a number of pathways including

the shikimic acid pathway via the penultimate gene, *isochorismate synthase* (*ICII*, also called *SID2*), in response to bacterial infection by *Pseudomonas syringae* pv. *maculicola* in *Arabidopsis* (Wildermuth et al., 2001). The transgenic plant, NahG contains a salicylate hydroxylase transgene that converts SA to catechol (a biologically inactive form of SA) (Friedrich et al., 1995). These plants accumulate little SA and display increased susceptibility to pathogens. *SIDI* encodes a multidrug and toxic compound exporter (MATE) transporter and may be involved in transporting SA out of the chloroplast (Nawrath et al., 2002), whereas *SID2* encodes a protein with isochorismate synthase activity and is involved in SA biosynthesis (Wildermuth et al., 2001). Neither mutant is able to accumulate SA and, as a result, little SA is present in the leaf.

Experiments manipulating the amount of SA present in the intercellular space revealed that when SA levels were reduced using salicylate hydroxylase, ARR was impaired. Conversely adding SA to the intercellular space enhances ARR in wild-type plants and restores ARR in *sid2*. SA has been demonstrated to be antimicrobial to *Pst* as adding 1mM SA to cultured *Pseudomonas* inhibited its growth (Cameron and Zaton, 2005). Increased intercellular SA during ARR also correlated to anti-bacterial activity in some cases (Cameron and Zaton, 2005). Together this data suggests that intercellular SA has antimicrobial properties thought to limit pathogen growth within the intercellular space (Cameron and Zaton, 2004).

To elucidate other genes required for ARR the Cameron lab employed a classical

mutant screen that identified *important for the ARR pathway (iap1-1)* (Carviel et al., 2009). At 6 weeks post germination (wpg) when wild-type Arabidopsis are ARR-competent, *iap1-1* plants display disease symptoms similar to young plants and other ARR defective mutants (NahG, *sid1*, *sid2*), including high levels of bacterial growth (10^7 cfu ml⁻¹) (Carviel et al., 2009). Genetic studies suggested that *iap1-1* is a semi-dominant mutation (Carviel et al., 2009). Basal resistance is not compromised in *iap1-1* plants as bacterial levels in young *iap1-1* and Col-0 are not significantly different (Carviel et al., 2009). An ARR defect in *iap1-1* was also demonstrated against *H. arabidopsidis* (Carviel et al., 2009). Infiltrating mature *iap1-1* with 0.1mM SA five hours prior to inoculation with 10^6 cfu ml⁻¹ *Pst* reduced pathogen growth by two-fold compared to plants infiltrated with water, suggesting that the *IAP1* gene is likely upstream of SA accumulation in the intercellular space (Carviel et al., 2009).

1.4.3 Arabidopsis-*Pst* System

Crop plants are difficult to study due to their long generation time, large size and frequent polyploidy (Lindeberg et al., 2008). *Arabidopsis thaliana* is a widely used plant model system because of its small size, 12 week generation time and small 120Mb fully sequenced genome (Theologis et al., 2000). *Arabidopsis thaliana* has the added benefit of being self-pollinating so the majority of offspring are homozygous and manual crosses can be performed for molecular and genetic studies.

For disease resistance studies *Arabidopsis* is a host to a number of diverse pathogens including bacteria (*Pseudomonas syringae* pv. *tomato* and pv. *maculicola*, *Xanthomonas campestris*, *Erwinia carotovora* and *Ralstonia solanacearum*), fungi (*Cladosporium* sp., *Alternaria brassicae*), oomycetes (*H. arabidopsidis*), viruses (cauliflower mosaic virus) and nematodes.

Pseudomonas syringae pv *tomato* (*Pst*) is a rod shaped, gram negative soil dwelling bacterium (Lindeberg et al., 2008). It can enter plant leaves via open stomata or wounds in the tissue (Lindeberg et al., 2008). Once inside *Pst* colonizes the intercellular space and uses the type III secretion system to deliver effectors into plant cells (Jones and Dangl, 2006). *Pst* is thought to obtain nutrients by causing plant cells to leak. *Pst* is an obligate biotroph depending on living plant tissue for the first part of its lifecycle and switches to necrotrophy (kills tissues and obtains nutrients from dead cells) later in its life cycle (Jones and Dangl, 2006).

Virulent *Pst* causes disease symptoms such as chlorosis, water soaked lesions and grows to high levels in the intercellular space of *Arabidopsis*. Mature plants (30 to 50 days post germination) inoculated with 10^6 cfu ml⁻¹ virulent *Pst* support a 10- to 100-fold reduction in bacterial growth compared to young plants given the same dose. Even young leaves of a mature plant display this resistance suggesting that ARR occurs in the entire plant. The *Arabidopsis-Pst* plant-pathogen system is also used to study another plant disease pathway: the Systemic Acquired Resistance (SAR) pathway.

1.5 Systemic Acquired Resistance (SAR)

SAR is a broad-spectrum response (Kuc, 1982). A primary, localized infection in one part of the plant results in partial or complete resistance to future infections in distant tissues to a number of other pathogens (Kuc, 1982). For example an initial infection with one class of organism, bacteria for example, results in resistance to not only other bacteria but also viruses and fungi (Kuc, 1982).

5.1.1 SAR occurs in distinct stages

Induction is the first stage of SAR, in which a pathogen infects one part of a plant leading to the second stage in which a long distance signal is generated and transmitted from the site of attack to distant tissues. Thirdly, the signal has to be perceived by the distant tissues such that the plant is primed and this is also called the establishment stage. The final stage, manifestation, occurs when the distant tissue is challenged by a pathogen and the plant responds in a resistant manner to a normally virulent pathogen (Champigny et al., unpublished).

A number of plant-pathogen models have been used to investigate the stages of SAR including tobacco-tobacco mosaic virus (TMV), Arabidopsis-*Pst* and cucumber-*Pseudomonas syringae* pv. *syringae* (*Pss*) (Wilcockson and Hull, 1974; Cameron et al., 1994; Stobel et al., 1996). The distant tissues of cucumber are SAR-competent one day after induction with *Pss* (Smith, Hammerschmidt and Fulbright, 1991). The initial leaf

must remain on the plant for six hours post inoculation but after that its detachment does not affect resistance in the rest of the plant (Smith, Hammerschmidt and Fulbright, 1991). *Pss* mutants unable to induce a hypersensitive response and systemic peroxidase activity in cucumber were also unable to elicit SAR (Smith, Hammerschmidt and Fulbright, 1991). In the Arabidopsis-*Pst* model developed by Cameron et al. (1994) one or two lower leaves are inoculated with *Pst* containing *avrRpt2* that is recognized by Arabidopsis R gene receptor RPS2, to initiate and establish a SAR response. Establishment typically takes two days at which point distant, uninoculated leaves can be challenged with virulent *Pst*. To determine if SAR has been established the amount of bacteria in the distant leaves is measured. Plants are either induced for SAR with *Pst* (*avrRpt2*) or mock-inoculated with MgCl₂. Two days later the distant leaves are challenged with virulent *Pst* and the *in planta* *Pst* levels of these distant leaves is measured after three days. If SAR-induced plants have two- to 10-fold less *Pst* than mock-inoculated plants, a SAR response was established. If SAR-induced and mock-inoculated plants have similar *Pst* levels then no SAR response was established.

SAR Induction

An initial infection by a SAR-inducing pathogen leads to a localized response that includes the formation of necrotic lesions, resulting from the hypersensitive response or a disease-causing infection (reviewed in Kuc 1982). The necrotic lesion triggers the expression of a set of pathogenesis-related (PR) proteins (Van Loon, 1999, Uknes *et al.*,

1992, 1993, Ward *et al.* 1991) and a 10- to 50-fold increase in SA over background levels (Delaney *et al.*, 1994, Lawton *et al.*, 1995, Malamy *et al.*, 1990, Uknes *et al.*, 1992, Yalpani *et al.*, 1991) in tobacco, cucumber and Arabidopsis (Kuc, 1982; Uknes *et al.*, 1992, 1993; Ward *et al.*, 1991).

Generation and Movement of the SAR Long Distance Signal(s)

Grafting experiments using cucumber provided evidence that a transmissible factor moves from the lower leaf induced for SAR to confer resistance in a distant upper leaf (Jenns and Kuc, 1979). Additional experiments suggested that a long distance signal(s) was moving via the phloem, as cucumbers girdled with hot cotton wool failed to establish SAR (Guedes *et al.*, 1980). Similar results were observed in tobacco plants when the stem sheath was removed (Tuzun and Kuc, 1985). It is important to note that both these techniques limit not only phloem movement, but also cell-to-cell movement down the petiole, therefore the SAR long distance signal could be traveling by either or both of these routes. Further evidence for phloem and cell-to-cell movement comes from Kiefer and Slusarenko (2003) who investigated SAR competence of individual leaves in relation to the source-sink resource allocation in the Arabidopsis rosette. As movement of the SAR signal occurs both inside and outside of the source-sink orthostichy, this suggests that the SAR long distance signal(s) is moving via the phloem and cell-to-cell.

The time it takes for the SAR signal to move and be perceived in distant tissue

varies by species. In the cucumber-*Pss* system the induced leaf must be left on the plant for approximately four hours, after which the induced leaf can be removed and SAR is still established in the distant tissues. This suggests that the long distance signal moves out of the induced leaf within four hours. It takes 24 hours for decreased susceptibility to be observed in the distant leaves of cucumber when SAR is induced using *Pss*, suggesting that the SAR signal moves and is perceived in distant tissues by 24 hpi. In the Arabidopsis-*Pst* system it takes 36-48 hours for the SAR signal to move and be perceived in distant tissues (Cameron et al., 1994). In tobacco it takes seven to nine days for signal movement and perception to be established (Park et al., 2007). This large range in establishment times suggests that cucumber is rapidly moving the SAR signal via the phloem, while Arabidopsis and tobacco may use a combination of phloem and cell-to-cell transport (Champigny and Cameron, In Press).

A number of molecules have been proposed to be SAR mobile signals or to be involved in long distance signaling. A SAR mutant screen identified *defective in induced resistance (dir1-1)*. The mutation in *dir1-1* was in DIR1, a putative lipid transfer protein (LTP) with a characteristic hydrophobic amino N-terminal signal sequence and eight cysteine residues held together by four disulfide bridges forming a large hydrophobic pocket in which lipids can bind. DIR1 is unique in the fact it is capable of binding two lipids side by side *in vitro* (Lascombe et al., 2008). Arabidopsis lacking DIR1 is SAR-defective as demonstrated by the lack of SAR-inducing activity in *dir1-1* petiole

exudates. Petiole exudates enriched in phloem sap were collected by immersing the cut petioles in 1mM EDTA to keep the sieve elements open. The exudates from a SAR-induced leaf of a wild-type plant can induce PR-1 expression if inoculated into a healthy, untreated wild-type plant (Maldonado et al., 2002). SAR-induced wild-type exudates can also elicit PR-1 gene expression in *dir1-1* tissues whereas neither SAR-induced or mock-inoculated *dir1-1* leaf exudates elicited PR-1 gene expression when infiltrated into wild-type plants (Maldonado et al., 2002). This suggests that DIR1 protein either produces an essential mobile signal or is transmitted from the induced leaf potentially serving as a chaperon for a lipid signal (Maldonado et al., 2002).

Further evidence that lipids play a key role in SAR comes from the identification of another mutant, *suppressor of fatty acid desaturase deficiency 1 (sfd1)* that has normal basal and R gene-mediated resistance but fails to establish SAR (Nandi et al., 2004). The *SFD1* gene encodes a dihydroxacetone phosphate reductase that is involved in plastid glycerolipid metabolism (Nandi et al., 2004). Similar to *dir1-1*, petiole exudates from *sfd1* did not contain SAR-inducing activity, as infiltrating petiole exudates from SAR-induced *sfd1* failed to induce SAR in naïve wild-type plants. However, a SAR-inducing activity was restored when exudates from SAR-induced *sfd1* were mixed with exudates collected from SAR-induced *dir1-1* plants suggesting that a plastid glycerolipid-dependent factor is required along with the DIR1-encoded lipid transfer protein (Chaturvedi et al., 2008). A SAR response in *sfd1* could be established if the mutant plants were inoculated with

SAR-induced wild-type exudates (Chaturvedi et al., 2008). An additional glycerolipid synthesis gene mutant Fatty Acid Desaturase 7 (*FAD7*) also lacks SAR-inducing activity and can be rescued with SAR-induced wild-type exudate (Chaturvedi et al., 2008). This suggests that *FAD7* may be involved in SAR long distance signaling while *SFD1* may be down stream of long distance SAR signaling.

Establishment

It is thought that the long distance signal(s) arrives in the distant tissue and then is perceived. How perception of the long distance signal(s) occurs is still unknown. It is thought that perception includes expression of PR proteins and accumulation of SA. It has been suggested that preception of the signal(s) 'primes' the distant tissue to respond quickly to a future invasion (Maldonado et al., 2002; Champigny et al., unpublished).

Manifestation

The final stage of a SAR response occurs when the distant tissues are challenged with a normally virulent pathogen and the plant responds with rapid resistance, including decreased *in planta* bacterial growth and symptoms. This resistance is accredited in part to the rapid accumulation of PR proteins. Once primed, plants will remain SAR-competent for varying lengths of time. Cucumber inoculated with *Colletotrichum lagenarium* was systemically protected from future *Colletotrichum lagenarium* invasions for four to five weeks. A second 'booster' inoculation extended the protection into the fruiting period. It took approximately 96 hours to establish protection but once initiated

the original leaf need not remain on the plant to maintain protection (Hammerschmidt, Acres and Kuc, 1976), suggesting that once the SAR signal has been perceived, establishment can be sustained without a continuous input of signal from the induced leaf.

Establishment of resistance can also vary within a species as cucumbers induced with *Pseudomonas syringae* pv. *lachrymans* take four to five days to establish resistance in distant tissue whereas infections with *Pseudomonas syringae* pv. *syringae* induce resistance after just one day (Smith, Hammerschmidt and Fulbright, 1991).

In the Arabidopsis-*Pst* system, SAR is induced by inoculating one or two lower leaves of four wpg plants with 10^6 cfu ml⁻¹ avirulent *Pst*. This primary infection produces a localized HR at the site of infection. For Arabidopsis it takes approximately two days for SAR to be established. If SAR is established, inoculation of distant leaves with 10^5 cfu ml⁻¹ virulent *Pst* will result in few disease symptoms and a two- to 10-fold reduction in bacterial growth. If SAR has not been established, *Pst* levels will not be reduced and leaves will display disease symptoms such as chlorosis and water soaking.

1.5.2 Role of Salicylic Acid in SAR

Salicylic acid was at one time thought to be a SAR signal molecule, but grafting experiments (Rasmussen et al., 1991; Friedrich et al., 1995) and SA-deficient transgenic plant studies (Gaffney et al., 1993; Pallas et al., 1996) suggest otherwise. Transgenic

tobacco expressing *nahG*, which encodes a salicylate hydroxylase gene accumulate very little SA and are SAR-defective when challenged with tobacco mosaic virus (TMV) (Friedrich et al., 1995). It also appears that SA is not a mobile signal in cucumber as demonstrated by the fact that the inoculated leaf only had to remain on the plant for four hours for SAR to be established in the distant tissue (Rasmussen et al., 1991). However, SA was not detectable in the petiole exudates of the induced leaves until eight hours after inoculation suggesting that SA is not the primary systemic signal of induced resistance in cucumbers (Rasmussen et al., 1991). However, SA still plays an important role in the distant tissues during SAR in tobacco as experiments with tobacco salicylate hydroxylase demonstrated decreased intercellular SA correlated to increased susceptibility (Delaney et al., 1994). In *Arabidopsis* accumulation of SA has been shown to be necessary for both local resistance which induces SAR and in distant tissues for priming and manifestation.

SA accumulation has been associated with the induction of PR genes in tobacco, cucumber and *Arabidopsis* (Malamy et al., 1990, Metraux et al., 1990 and Delaney et al., 1994). In tobacco, a 20-fold increase in SA is observed in tissues infected with tobacco mosaic virus (TMV) and a five-fold increase in systemic tissues (Malamy et al., 1990). In *Arabidopsis* 10- to 50-fold increases in SA have been observed in tissues after infection with a virulent strain of *Pseudomonas syringae* pv. *tomato*. SA also accumulates in the distant tissue of infected plants (Zhou et al., 1998, Cameron et al., 1999).

In systemic tissues as part of the establishment stage, SA accumulation leads to

the reduction of disulfide bonds in NPR1 allowing it to enter the nucleus where it can interact with TGA transcription factors that leads to the expression of PR proteins. *non expressor of PR gene 1 (npr1)* mutants accumulate SA but have reduced PR gene expression and have enhanced susceptibility to various pathogens. NPR1 interacts with transcription factors that bind to elements of the PR-1 promoter. Therefore, *NPR1* is thought to be required in distant tissues during the establishment of SAR, but not the induced leaf as the RPS2-avrRpt2 interaction that induces SAR does not require functional *NPR1* (Bowling et al., 1997)

1.5.3 Potential Mobile Signals

Jasmonates (Jasmonic Acid [JA] and its derivatives including Methyl JA)

A number of JA mutants including *sgt1b* (*suppressor of the G2 allele of skp1*), *opr3* (*isozyme of 12-oxophytodienoate reductase*) and *jin1* (*jasmonate insensitive*) (Kiba et al, 2003; Stintzi and Browse, 2000) all have shown SAR-deficient phenotypes suggesting that JA could play a major role in SAR (Truman et al., 2007). Comparison of genes that are active during basal defense and those up-regulated after inoculation with avirulent pathogens revealed a number of JA biosynthetic pathway genes suggesting that the JA pathway may be involved in plant disease defense (Truman et al., 2007). SAR-induced petiole exudates collected from *Fatty Acid Desaturase7 (FAD7)*, which

synthesizes the fatty acids required for jasmonic acid biosynthesis, were unable to provide protection in distant tissues of wild-type plants when challenged with virulent pathogen, suggesting that JA may be involved in the SAR pathway. However, co-infiltrating exudates from SAR-induced *fad7* with JA did not restore SAR activity suggesting that JA is not the SAR mobile signal (Chaturvedi et al., 2008). The JA signaling pathway cross-communicates with SA-dependent pathways (such as a SAR) often acting in an antagonistic fashion (Pozo, Van Loon and Pieterse, 2004). For example, if JA is produced during a plant-pathogen interaction then SA may be suppressed such that many SA-dependent defenses including basal defense will not be expressed (O'Donnell et al, 2003).

Methyl Salicylate (MeSA)

Salicylic acid-binding protein 2 (SABP2) is an esterase that is thought to convert biologically inactive MeSA to active SA in tobacco. The AtMES family of proteins are functional homologs of SABP2 in Arabidopsis (Kumar et al., 2005). SABP2 also binds SA with high affinity suggesting that negative feedback regulates SABP2 methyl esterase activity. Grafting experiments in tobacco demonstrated that SABP2 is required in the systemic leaves for SAR to occur. Further studies using mutant SABP2 proteins suggested that SABP2 esterase activity is critically important in the systemic leaf. These studies also suggested that SA-mediated inhibition of SABP2 in the induced leaf was necessary to allow a critical amount of MeSA to accumulate and that MeSA moves to

distant tissues as a SAR long distance signal, where it is converted to SA by the esterase activity of SABP2.

Azelaic Acid

Jung et al. (2009) recently reported that induction of SAR in Arabidopsis by avirulent *Pst* causes accumulation of azelaic acid, a mobile metabolite that accumulates in the petiole exudates. Mutation of the *Azelaic Acid Induced 1 (AZII)* gene, which is induced by azelaic acid, results in a SAR-defective plant. Azelaic acid accumulated in the petiole exudates of SAR-induced leaves more than in mock-inoculated leaves suggesting movement. Together these data led the authors to suggest that azelaic acid may also be a SAR long distance signal.

There are currently a few unsolved issues in the SAR literature. Attaran et al. (2009) provide evidence suggesting that neither MeSA or JA are essential SAR signals. The ability to produce MeSA and the establishment of SAR do not coincide in a number of mutants. For example pathogen-responsive SA methyltransferase mutant *bsmt1* cannot produce detectable levels of MeSA, but is still SAR-competent. However, very low levels of MeSA may be enough to trigger a small SAR response. JA biosynthesis mutants *dde2*, *opr3* and downstream JA signal mutants *coi1*, *jar1* and *jin1* are all SAR-competent under different laboratory conditions suggesting that there maybe an environmental component to JA's involvement in SAR (Cui et al., 2009).

DIR1

The *DIR1* gene encodes a putative lipid transfer protein (LTP) with a hydrophobic amino N-terminal signal sequence and eight cysteine residues common to all LTPs. The crystal structure of DIR1 was recently determined by x-ray diffraction and fluorescence spectroscopy. DIR1 falls into a novel class of LTPs as it has the potential to bind two lipids side by side in a large internal hydrophobic pocket (Lascombe et al., 2008). Mutant *dir1-1* plants exhibit wild-type resistance in the local leaf suggesting that basal defense is not affected, but fail to develop SAR (Maldonado et al., 2002). Distant *dir1-1* leaves induced for SAR express little PR-1 suggesting that DIR1 is either involved in the signal generation and transmission stage or is required in the distant tissues as part of the establishment stage (Maldonado et al., 2002). Petiole exudates (enriched for phloem sap) from the leaf of a SAR-induced wild-type plant can induce PR-1 gene expression when infiltrated into naïve *dir1-1* leaves (Maldonado et al., 2002). However SAR-induced *dir1-1* petiole exudates can not induce PR-1 gene expression in naïve wild-type leaves (Maldonado et al., 2002). These data suggest that DIR1 functions in the signal generation or transmission stage of SAR. The presence of a signal sequence suggests that DIR1 is targeted to the cell wall (Maldonado et al., 2002).

1.5.4 Localization and Expression of DIR1

RNA and protein gel blot experiments demonstrated that DIR1 is constitutively expressed at low levels in leaves and that DIR1 expression is reduced after plants are induced for SAR (Maldonado et al., 2002). Later studies using type III secretion system mutants indicate that this reduction is likely due to pathogen-mediated suppression of DIR1 and is not a plant response (Champigny et al., unpublished). The type III secretion system (T3SS) of *Pst* delivers virulence effectors, some of which have previously been demonstrated to suppress the expression of a number of Arabidopsis genes including lipid transfer proteins (Jones et Dangl, 2006). Leaves collected from wild-type plants inoculated with *hrpS Pst* (a T3SS mutant incapable of delivering virulence effectors into plant cells) displayed a steady increase in DIR1 expression from three to 18 hpi in leaves when examined by RNA gel blots (Champigny et al., unpublished). In contrast, DIR1 expression was greatly reduced from three to 18 hpi in leaves inoculated with wild-type virulent *Pst* (Champigny et al., Unpublished). Taken together this data suggests that DIR1 expression is suppressed by *Pst* during SAR.

Unpublished work from the Cameron lab further defined the location of DIR1 during SAR. DIR1 was hypothesized to be expressed in the companion cells of the phloem because this would give it access to sieve elements for movement to distant leaves and this would also explain the observation that DIR1 is expressed at low levels. To investigate if DIR1 is expressed in the phloem, DIR1 expression was visualized in

plants using the β -glucuronidase (GUS) reporter gene. Two transgenic plant lines were created, one contained the DIR1 promoter region upstream of GUS in the wild-type Ws-0 background (DIR1:GUS) and the other contained the DIR1 promoter plus DIR1 coding sequence fused to GUS in the *dir1-1* mutant background (DIR1pro:DIR1-GUS). At four weeks post germination both lines were examined before and during SAR. Leaves were collected from untreated, mock-inoculated and SAR-induced leaves 14 hours post inoculation. Abundant GUS staining was observed in the vasculature and mesophyll cells of untreated and mock-inoculated leaves from both plant lines. Less intense GUS staining was observed in leaves inoculated with SAR-inducing *Pst (avrRpt2)*. Untreated systemic leaves from the SAR-induced plants of both transgenic lines also had less intense GUS staining. This provides evidence that DIR1 appears to be expressed in all cell types including the vasculature in the leaves and expression may be reduced after SAR-induction (Champigny et al., unpublished).

To obtain a better view of cellular DIR1 expression, GUS-stained leaves from the DIR1pro:GUS and DIR1pro:DIR1-GUS transgenic lines were sectioned and viewed under high magnification. GUS activity was observed in all cell types including the xylem, xylem parenchyma and phloem (companion cells and sieve elements). DIR1 was expressed in all cell types of leaves induced for SAR, suggesting that DIR1 is expressed in the phloem both before and during SAR and thus has access to the phloem for long distance signaling (Champigny et al., Unpublished).

To localize DIR1 within the cell, *Nicotiana tabaccum* plants were transformed with *Agrobacterium* containing a T-DNA with full-length DIR1 fused to a reporter gene, enhanced yellow fluorescent protein (35S:DIR1-EYFP). *Agrobacterium* is an efficient way to transform tobacco cells to see where a protein is targeted in the cell (Champigny et al., unpublished). Microscopy of transformed leaves revealed that DIR1 was targeted to the cell wall. This localization appears to be mediated by a short signal sequence as tobacco transformed with a truncated DIR1 that lacked the signal sequence fused to EYFP (35S:DIR1-ss-EYFP) localized to the cell periphery and in cytoplasmic streams (Champigny et al., unpublished).

To demonstrate similar targeting of DIR1 in Arabidopsis, transgenic lines of DIR1 without the signal sequence fused to GUS were created in a *dir1-1* mutant background. Intercellular washing fluids (IWFs) consist of cell wall associated proteins and provide information about the soluble proteins associated with the plant cell walls. IWFs from DIR1-GUS/*dir1-1* and wild-type Ws-0 plants contain DIR1 signal when examined by protein gel blot analysis. Mutant *dir1-1* plants did not have any DIR1. DIR1 without the signal sequence was not present in the IWF suggesting that the signal sequence is required to target DIR1 to the cell wall. (Champigny et al., unpublished).

1.5.5 The Discovery of DIR1-like

Two experimental observations in the Cameron lab led to the suggestion that a DIR1-like protein exists in Arabidopsis. Exudate experiments involve collecting petiole exudates from the cut petiole ends of Arabidopsis. DIR1 is found in the petiole exudates of wild-type SAR-induced plants, but typically not in *dir1-1*. In some of the exudate-protein gel blot experiments, a DIR1 signal was detected in SAR-induced *dir1-1* plants. Secondly *dir1-1* occasionally (two of 30 experiments at University of Toronto and three of 16 at McMaster over a total of 11 years) displayed a partially SAR-competent phenotype. After loss of the T-DNA insert in the *dir1* gene was ruled out as the cause by RT-PCR, we speculated that a DIR1-like gene may in some instances compensate for the SAR defect in *dir1-1*. A BLAST search with DIR1 found At5g48490 which is located adjacent to DIR1 (At5g48485) on chromosome five. The two coding sequences shared 71% sequence identity and 81% sequence similarity at the amino acid level. The majority of variation between the two sequences occurs in the ER signal sequence which targets the immature protein to the ER and is then cleaved. The mature proteins (minus the signal sequence) share 88% amino acid similarity (Champigny et al., unpublished).

Real time PCR indicated that *DIR1-like* is expressed at similar levels in wild-type plants. *DIR1-like* also appears to be suppressed by *Pst* during SAR induction. *DIR1* and *DIR1-like* are less than 1000 kb apart on chromosome five so there was concern the T-DNA insert in *DIR1* may affect upstream binding motifs in the promoter of *DIR1-like*

(Champigny et al., unpublished). *DIR1-like* expression in *dir1-1* plants is comparable to *DIR1-like* expression in wild-type plants indicating that it is not affected by the silenced *DIR1* gene (Champigny et al., unpublished). Homology modeling indicates a potentially key amino acid change in the internal lipid binding cavity of *DIR1-like*. In *DIR1* residue 40 is a phenylalanine, whereas *DIR1-like* has a tyrosine (Champigny et al., unpublished). This change may affect the ability of *DIR1-like* to bind lipids.

1.6 Similarities and Differences between ARR and SAR

SA is important for both ARR and SAR. For the SAR pathway, SA is needed in the distant leaf for establishment and manifestation of SAR (Cameron et al., 1999). For the ARR pathway SA accumulation is also required (Cameron and Zaton, 2005). However, they are distinctively different pathways as SAR requires *npr1* expression in the distant leaf while ARR does not. In the SAR pathway SA appears to be a signaling molecule, although not the long distance signal, activates *NPR1* and TGAs leading to *PR-1* expression in distant leaves. In ARR, *NPR1* and *PR-1* function are not required, instead SA is required in the intercellular space, potentially serving an antimicrobial role.

Research Objectives / Rational

The main goal of the work described in this thesis is to contribute to the elucidation of plant disease defense pathways particularly the Age Related Resistance (ARR) pathway and the Systemic Acquired Resistance (SAR) pathway using the *Arabidopsis-Pseudomonas syringae* pv. *tomato* pathosystem.

Objective 1:

Develop a robust mutant screening method to obtain ARR-defective mutants.

Objective 2:

Determine the relationship between *JIN1* and *DIR1* during SAR.

Objective 3:

Obtain definitive evidence that DIR1 is a long distance signal during SAR by following its movement.

Objective 4:

Develop a Cucumber-Arabidopsis SAR model to

a) aide in SAR studies.

b) provide evidence that a DIR1 orthologue exists in cucumber.

Materials and Methods

Plant Growth Conditions

Arabidopsis thaliana Growth Conditions

A summary of all the *Arabidopsis thaliana* ecotypes, mutant and transgenic lines used in this thesis is provided below in Table 1. Columbia-0 (Col-0), Wassilewskija (Ws-0), *defective in induced resistance1-1 (dir1-1)*, *jasmonate insensitive 1 (jin1)*, DIR1 overexpression line (35S:DIR1 in *dir1-1* 5E), *non-expressor of pathogenesis-related genes 1-2 (npr1-2)* and DIR1 antisense line (35S:antisenseDIR1-3B) were used for SAR experiments. Col-0, *salicylic acid induction deficient 2 (sid2)* and *important for the ARR pathway 1-1 (iap1-1)* were used for the ARR fluorescence assay experiments.

Table 1: *Arabidopsis* ecotypes and mutants used throughout this thesis.

<i>Name</i>	<i>Description</i>	<i>Source</i>
Columbia-0 (Col-0)	Wild-type	Arabidopsis Biological Resource Center (ABRC) – Ohio State University
Wassilewskija (Ws-0)	Wild-type	Arabidopsis Biological Resource Center (ABRC) – Ohio State University
<i>defective in induced resistance1-1 (dir1-1)</i>	SAR-deficient. Ws-0 background.	(Maldonado <i>et al.</i> , 2002)
35S:DIR1 in <i>dir1-1</i> 5E	Overexpressing line of DIR1	Arabidopsis Biological Resource Center (ABRC) – Ohio State University

<i>non-expressor of pathogenesis-related genes 1-2 (npr1-2)</i>	SAR-deficient. Col-0 background.	Arabidopsis Biological Resource Center (ABRC) – Ohio State University
35S:antisense DIR1 in <i>dir1-1</i> 3B	SAR-deficient. Anti-sense DIR1. Ws-0 background.	(Maldonado <i>et al.</i> , 2002)
<i>salicylic acid induction deficient 2 (sid2)</i>	ARR-deficient. Col-0 background. Can not accumulate salicylic acid (SA).	(Dewdney <i>et al.</i> , 2000)
<i>important for the ARR pathway 1-1 (iap1-1)</i>	ARR-deficient. Col-0 background.	(Carviel <i>et al.</i> , 2009)
<i>jasmonate insensitive 1 (jin1)</i>	MYC family transcription factor involved in mediating a subset of jasmonate induced responses.	(Laurie-Berry <i>et al.</i> , 2006) B. Kunkel (Washington State)

Seeds were surface sterilized in 70% ethanol for two minutes, followed by 10 minutes of occasional shaking in seed sterilization solution (0.018% bleach, and 0.1% Tween-20), then rinsed five times in sterile dH₂O, suspended in 0.1% phytagar (Caisson Laboratories Phytoblend) and vernalized at 4°C for at least two days before plating on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) followed by germination under continuous light. Seedlings were then transferred after five to seven days to soil (Sunshine Mix No. 1 - Jack Van Klaveren, Ste Catherines, Ontario) moistened with 1 g l⁻¹ 20-20-20 fertilizer and grown until three weeks of age (ARR Young Plant Experiments), four weeks of age (SAR Experiments) or six weeks of age (ARR Mature Plant Experiments). The temperature was maintained between 22°C and 24°C under a

nine-hour photoperiod with an average light intensity of $150 \mu\text{Em}^{-2} \text{sec}^{-1}$. The first 48 hours post transplant to soil the seedlings were covered with a plastic dome to maintain high humidity (~100%). After the dome was removed humidity ranged between 70 and 85%. All plants were watered as required (when tray was empty) with dH_2O . Plants used in ARR experiments were fertilized again with 1 g l^{-1} 20-20-20 fertilizer at two and five weeks post germination.

Cucumis sativus (Cucumber) Growth Conditions

Cucumber Wisconsin S.M.R. 58 146B seeds (Stokes Seeds LTD., Ste. Catharines, Ontario) were planted directly into soil (Sunshine Mix No. 1 - Jack Van Klaveren, Ste. Catharines, Ontario). Seeds were placed approximately three centimeters into soil and covered. Soil was hydrated with 1 g l^{-1} 20-20-20 fertilizer and plants were grown for 21-23 days at 22°C to 28°C , under a 16 hour photoperiod at $150 \mu\text{E m}^{-2} \text{s}^{-1}$ light intensity and 75 to 85% relative humidity. All plants were watered as required (when tray was empty) with dH_2O .

Bacterial Growth Conditions

Virulent (containing pVSP61) and avirulent (containing pVSP61 + *avrRpt2*) *Pseudomonas syringae* pv. *tomato* DC3000 strains are previously described (Whalen et al., 1991). Cucumber pathogen *Pseudomonas syringae* *syringae* D20 is a virulent

cucumber pathogen previously described (Smith, Hammerschmidt and Fulbright, 1991).

Pseudomonas syringae pv. *tomato* DC3000 pDSK-GFPuv is a virulent *Pseudomonas* strain with GFP introduced on a stable plasmid (Wang *et al.*, 2007). *Agrobacterium tumefaciens* GV3101 was used to transiently express 35S:EYFP, 35S:DIR1-EYFP or 35S:DIR1-like (Champigny *et al.*, unpublished).

Table 2: Bacterial strains used in this thesis

Name	Description	Antibiotic Resistance	Medium	Source
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000 pVSP61	Virulent pathogen to <i>Arabidopsis</i>	Rifampicin (100 µg ml ⁻¹) [chromosomal], Kanamycin (50 µg ml ⁻¹) [plasmid]	King's B	Dr. Andrew Bent (University of Wisconsin at Madison) (Whalen <i>et al.</i> , 1991)
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000 pV288 (pVSP61 + <i>avrRpt2</i>)	Avirulent on <i>Arabidopsis</i>	Rifampicin (100 µg ml ⁻¹), Kanamycin (50 µg ml ⁻¹)	King's B	Dr. Andrew Bent (University of Wisconsin at Madison) (Whalen <i>et al.</i> , 1991)
<i>Pseudomonas syringae syringae</i> D20	Avirulent on Cucumbers		King's B	Dr. Ray Hammerschmidt (University of Michigan)
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000 pDSK-GFPuv	Virulent pathogen to <i>Arabidopsis</i> Enhanced GFP	Rifampicin (100 µg ml ⁻¹), Kanamycin (50 µg ml ⁻¹)	King's B	(Wang <i>et al.</i> , 2007)

<i>Agrobacterium tumefaciens</i> GV3101 harbouring a 35S:EYFP binary expression vector	Transient expression of EYFP	Rifampicin (50 $\mu\text{g ml}^{-1}$), Streptomycin (75 $\mu\text{g ml}^{-1}$), Gentamycin (100 $\mu\text{g ml}^{-1}$)	Yeast Peptone Media	(Champigny et al., unpublished)
<i>A. tumefaciens</i> GV3101 harbouring a 35S:DIR1-EYFP binary expression vector	Transient expression of DIR1-EYFP	Rifampicin (50 $\mu\text{g ml}^{-1}$), Streptomycin (75 $\mu\text{g ml}^{-1}$), Gentamycin (100 $\mu\text{g ml}^{-1}$)	Yeast Peptone Media	(Champigny et al., unpublished)
<i>A. tumefaciens</i> GV3101 35S:DIR1-like	Transient expression of DIR1-like	Rifampicin (50 $\mu\text{g ml}^{-1}$), Streptomycin (75 $\mu\text{g ml}^{-1}$), Gentamycin (100 $\mu\text{g ml}^{-1}$)	Yeast Peptone Media	(Champigny et al., unpublished)

ARR Inoculations

Virulent *Pseudomonas syringae* pv. *tomato* DC3000 was grown overnight with agitation (200rpm) until it reached mid-log phase (~16 hours at room temperature [22-25°C]) in King's B media with kanamycin (final concentration 5 $\mu\text{g}/\mu\text{l}$). Cultures were diluted to 10⁶ colony forming units per ml (cfu ml⁻¹) in 10mM MgCl₂. This diluted pathogen was pressure infiltrated into the abaxial surface of three to five *Arabidopsis* leaves per plant with a needleless syringe.

In planta bacterial density was determined three days later by isolating *Pseudomonas syringae* from infected leaves by shaking leaf discs in sterile 0.2% silwet in

10mM MgCl₂. Silwet is a detergent that interrupts cell membranes allowing intercellular bacteria to be released into solution. Leaves were clipped from the plant, surface sterilized (50% ethanol, 49% Sterile dH₂O, 1% Bleach), and then rinsed in sterile dH₂O). Leaf discs were collected from each side of the mid-vein in the middle of the leaf using a four millimeter cork bore. Eight leaf discs were shaken in 1ml of 10mM MgCl₂ + 0.2% silwet for one hour. After one hour of shaking in the silwet solution a dilution series was made from the wash solution and plated on King's B with kanamycin and rifampicin. The resulting colonies were counted and the following formula was applied to determine cfu ml⁻¹: Colony Count x Dilution Factor x Plating Multiplier* / # of Leaf Discs. The plating multiplier is volume diluted compare to total volume. For example if 10µl of the 1000µl leaf disc solution was serially diluted the plating multiplier would be 100.

ARR-GFP Screen

Mature *Arabidopsis* (six weeks post germination) were inoculated as described above except virulent *Pseudomonas syringae* pv. *tomato* DC3000 pDSK + GFPuv was used as inoculum. *Pst* pDSK + GFPuv constitutively expresses enhanced GFP. Col-0 and *iap1-1* were used for these experiments.

In planta bacterial density was determined as described above. After one hour 200µl of sample was placed in a Costar black, flat bottom 96-well microtiter plate and fluorescence was measured in an Xfluo4 SAFIRE plate reader (excitation wavelength = 485nm, emission wavelength = 535nm).

Use of *Agrobacterium* to Transiently Express Genes

Agrobacterium tumefaciens harbouring a 35S:EYFP or 35S:DIR1-EYFP binary expression vector were grown over two nights with agitation (200rpm) at room temperature (22-25°C) in yeast peptone media with streptomycin (final concentration 150µg/ul), rifampicin (final concentration 75µg/µl) and gentamycin (final concentration 105µg/µl) . Cultures were diluted to either an optical density of 0.4 or 10⁶ colony forming units per ml (cfu ml⁻¹) in 10mM MgCl₂. This diluted pathogen was pressure infiltrated into the abaxial surface of two to three lower leaves of each *Arabidopsis* plant with a needleless syringe. *Agrobacterium* mediated expression of the gene of interest takes four days.

SAR Assay

SAR-inducing *Pst* DC3000 pV288 (pVSP 61 + *avrRpt2*) was grown overnight with agitation (200rpm) until mid-log phase (~16 hours at room temperature, 22-25°C) in King's B media with kanmycin (final concentration 5µg/ul). Cultures were diluted to 10⁶ colony forming units per ml (cfu ml⁻¹) in 10mM MgCl₂. This diluted pathogen was pressure infiltrated into the abaxial surface of 2-3 lower leaves of each *Arabidopsis* plant with a needleless syringe. For *Agrobacterium*-SAR experiments the same 2-3 leaves that received an *Agrobacterium* inoculation were inoculated with SAR-inducing *Pst* 4 days after the *Agrobacterium* infiltration.

Virulent *Pst* DC3000 was grown overnight with agitation (200rpm) until mid-log phase (~16 hours at room temperature 22-25°C) in King's B media with kanimycin (final concentration 5µg/ul). Cultures were diluted to 10^5 colony forming units per ml (cfu ml⁻¹) in 10mM MgCl₂. This diluted pathogen was pressure infiltrated into the abaxial surface of three to five upper, naïve *Arabidopsis* leaves per plant with a needleless syringe. Three days later *Pst* levels were quantified as described above.

It is important to differentiate between a SAR Assay and an Agro-SAR Assay. An Agro-SAR assay begins when plants are approximately three and a half wpg at which time they receive the first of three inoculations. The plants are first inoculated on one or two lower leaves with *Agrobacterium* with the gene of interest, four days later they receive a second inoculation on the same leaf to induce SAR and the final inoculation comes two days later when uninoculated leaves are challenged with virulent *Pst*. A SAR Assay consists of only two inoculations and typically begins when plants are three and half to four wpg. At approximately four weeks one or two lower leaves are either SAR-induced with *Pst(avrRpt2)* or mock-inoculated, two days later uninoculated leaves are challenged with virulent *Pst*. After 48 hours the resulting *Pst* colonies can be counted with the aid of a colony counter. The number of colony forming units (cfu) per leaf disc was calculated by multiplying the number of colonies by the dilution factor (10^4) and the plating factor (100µl of 1ml) and dividing by the total number of leaf discs in the wash (eight).

Cucumber-Arabidopsis SAR Assay

Pseudomonas syringae syringae D20 was grown overnight with agitation (200rpm) until mid-log phase (~16 hours at room temperature 22-25°C) in King's B media. Cultures were diluted to 10^7 colony forming units per ml (cfu ml⁻¹) in 10mM MgCl₂. This diluted culture was pressure infiltrated into the abaxial surface of the lowest two cucumber leaves. Approximately 30 inoculations were made in each leaf (approximately five on each side of the mid-vein).

***dir1-1* Rescue Using Cucumber Exudate**

Cucumber exudate was collected by cutting the petiole on an angle three to five centimeters below the leaf with a razor blade. 30 to 40µl of exudate was collected from cut ends using 20µl glass capillary (VWR). Exudate was immediately diluted into 300µl of cucumber exudate buffer (0.05M Tris-HCl, pH 7.5 with 0.1% β-Mercaptoethanol). Samples were either stored at 4°C awaiting infiltration into *Arabidopsis* leaves for cucumber SAR assay or frozen at -20°C for later concentration and immunoblotting.

Arabidopsis Petiole Exudate Collection

Petiole exudates were collected as previously described by Maldonado *et al.*, 2002. Briefly, phloem enriched petiole exudates were collected by cutting one petiole at a time just above the stem. Leaves were surface sterilized for 10 seconds in 50% ethanol,

0.0006% bleach, then rinsed in 1mM sterile EDTA. Seven to ten leaves were collected per tube followed by submerging the cut ends in ~1.5ml of 1mM EDTA to prevent callose formation. Tubes were kept in a moist, high humidity environment while exuding over 48 hours at room temperature. Samples were immediately frozen at -20°C until concentrating and immunoblotting. Cucumber petiole exudates were lyophilized to >1µl then reconstituted in 100µl sterile dH₂O. *Arabidopsis* petiole exudates were lyophilized to >1µl then reconstituted in 20-60µl sterile dH₂O.

Total Protein Extraction from Leaves

Agrobacterium infiltrated leaves were collected four days post infiltration, flash frozen in liquid nitrogen and stored at -80°C. Total protein was extracted from the frozen leaves by grinding the tissue to a fine powder with a hand held drill and plastic bit in 150-250µl of chilled RIPA buffer (50mM Tris-HCl; pH 7.5, 1mM EDTA, 100mM NaCl, 1% NP-40, 0.1% SDS, 0.1% Triton X-100, 0.7% β-mercaptoethanol, 1mM PMSF) followed by end-over-end agitation at 4°C for 30 minutes. Samples were centrifuged at 15,300 rpm for five minutes to pellet debris. Supernatant protein concentrations were determined by the Bradford assay (Bradford, 1976).

Protein Gel Blot Analysis with anti-DIR1 antibody

16µl of exudate was denatured in 4µl of 6X SDS loading buffer (350mM Tris-

HCl; pH 6.8, 30% glycerol, 10% SDS, 0.01% bromophenol blue, 5mM DTT) in a 95°C water bath for five minutes. DTT was increased to 200mM for some experiments and is noted as such in the results. Samples were loaded on a precast 4-12% NuPAGE Bis-Tris gel (Invitrogen) and run in 1X MES SDS running buffer (50mM MES, 50mM Tris, 3.5mM SDS, 1mM EDTA; pH 7.3-7.7) using the Xcell Surelock protein rig. Five ng of LTP was used as a positive control. SeeBlue Plus2 ladder (Invitrogen) was used as the molecular marker. Gels ran at 70V for 2.5 hours.

Semi-dry transfer to nitrocellulose membrane

Gels were soaked in Towbin transfer buffer (25mM Tris base, 192mM glycine, 20% methanol) for 20 minutes with one refreshment to remove residual SDS. Proteins were transferred from the gel to a Protran nitrocellulose membrane (Schleicher and Schuell) between two pieces of extra thick blotting paper soaked in Towbin transfer buffer (25mM Tris base, 192mM glycine, 20% methanol) at 9-11V for 30 minutes using BioRad semi-dry transfer apparatus.

1° antibody incubation

Membranes were washed in 1X Tris buffered saline with Tween-20 (TBST – NaCl, KCl, Tris base; pH 7.4, 0.05% Tween-20) for five minutes to remove residual methanol and then blocked for 1 hour in 5% milk in TBST. Membrane was probed with either 1:20000 α DIR1 polyclonal antibody or 1:10000 α EGFP in 5% milk (Nestle Carnation Instant Skim Milk Powder) in TBST for 16 hours at 4°C with gentle agitation.

2^o antibody incubation

After 1^o antibody incubation the membrane was washed for 50 minutes with five exchanges of TBST at room temperature with gentle shaking and then incubated with 1:3000 goat anti-rabbit HRP 2^o antibody (Pierce Supersignal West Femto kit) for two hours at room temperature (25°C) with gentle agitation.

Detection

Membrane was detected with Westfemto peroxide – luminal kit (Pierce) and BioMax XAR film (Kodak). Film was exposed for two minutes and developed. Long term exposures were also developed at 48 hours post detection.

RNA Isolation, RNA Quantification & cDNA synthesis

Leaves inoculated with *Agrobacterium* were collected four days post infiltration, flash frozen in liquid nitrogen and stored at -80°C. RNA was extracted from leaf samples using the TRIzol method, according to the manufacturer's instructions (Invitrogen). DNase treatment was performed with the DNase Free (Ambion) system, according to the manufacturer's instructions. RNA was quantified using a spectrophotometer. cDNA was synthesized using the SuperScript III (Invitrogen) reverse transcriptase kit, according to the manufacturer's instructions.

Reverse-Transcription PCR

Each set of gene specific primers were optimized to 54°C. Tests were carried out for each set of gene-specific primers (Table 3) to determine the cycle number giving logarithmic amplification. Primers were synthesized by Mobix. For the genes analyzed in this study, 28 cycles of PCR were found to give amplification in the logarithmic range. Actin was used as the constitutive internal control, optimized to 61°C and 28 cycles. Primers were designed to amplify across the gene of interest-YFP fusion junction. Note: primer DYfusion3p sits in the YFP side of the fusion and was used for both DIR1-EYFP and DIR1Like-EYFP. After constructing DIR1Like-EYFP primers it was noted the *Agrobacterium* DIR1-like strain did not contain EYFP as DIR1-like was discovered after the tobacco YFP experiments.

Table 3: Primers used in this thesis:

DYfusion5p	5'-GGTGTTGATCCTGAACTCGC-3'
DYfusion3p	5'-AACTTCAGGGTCAGCTTGCC-3'
DLYfusion5p	5'-AAGCTCGCTTCTAGTCTCCC-3'
ActinR	5'-GGTCACGACCAGCAAGATCAAGACG-3'
ActinF	5'-GGCGATGAAGCTCAATCCAAACG-3'

Results

Systemic Acquired Resistance

1. DIR1 is detected in petiole exudates of SAR-induced Col-0

Previous experiments in the Cameron lab have demonstrated that DIR1 is present in petiole exudates collected from wild-type Ws-0 Arabidopsis induced for SAR, suggesting that DIR1 moves down the petiole after SAR-induction (Champigny et al., unpublished). If DIR1 is part of a SAR signal translocation complex, then it should be possible to detect DIR1 in the petiole exudates of other Arabidopsis ecotypes as it should be transmitted to distant tissues to prime them for SAR. To determine if DIR1 moves down the petiole in a similar fashion in another wild-type Arabidopsis ecotype, Col-0 and to determine how long after SAR-induction DIR1 begins to travel down the petiole, the following experiment was performed. SAR-competent Col-0 leaves were either mock-inoculated or induced for SAR with *Pst(avrRpt2)*, cut from the plant at specific time intervals (17, 23, 29 hours post inoculation [hpi]), quickly surface sterilized and then immersed cut end down in 1mM EDTA which prevents the sieve elements from closing (King and Zeevaart, 1974). Typically seven leaves were pooled to make one exudate. The samples of both SAR-induced and mock-inoculated exudates collected at 17 hpi and 23 hpi exuded for six hours (until 23 hpi and 29 hpi respectively) and the samples cut from the plants at 29 hpi exuded for 15 hours (until 44 hpi). All petiole exudates were concentrated by lyophilization and subjected to protein gel blot analysis with anti-DIR1

antibody.

A DIR1 signal of approximately 15 kDa was observed in exudates collected from SAR-induced Col-0 (Figure 2, Lanes 1 -9), but nothing was observed in mock-inoculated samples (Figure 2, Lane 10), suggesting that DIR1 protein is not present at detectable levels in exudates prior to SAR induction.

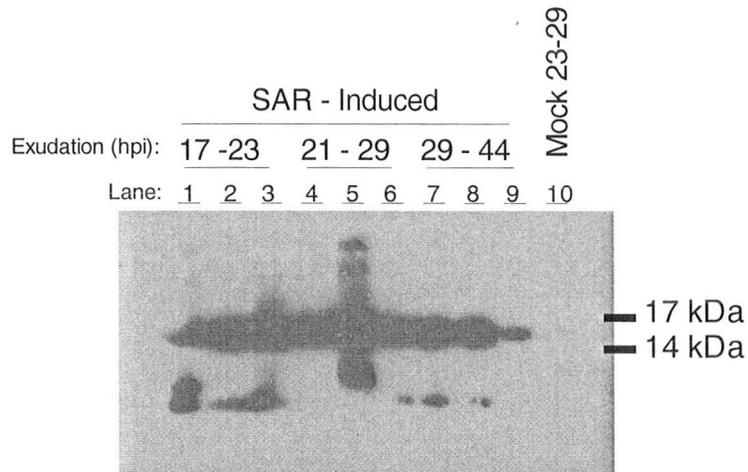


Figure 2: Anti-DIR1 protein gel blot of petiole exudates from SAR-induced and mock-inoculated Col-0. Petioles were removed and exuded for various lengths of time starting at 17-23, 23-29 and 29-44 hpi for SAR-induced samples. Mock-inoculated petiole exudates were collected 23-29 hpi. Mock-inoculated petiole exudates collected 17-23 and 29-44 hpi also had no product (data not shown). This experiment was repeated twice more with similar results (Appendix 1, Figure 17)

JIN1 and DIR1 in the SAR pathway

Work in the Cameron lab, including the previous experiment, suggests that DIR1 moves down the petiole after SAR induction (Champigny et al., unpublished). Recently the Jasmonic Acid (JA) pathway has been implicated in the SAR response by other

research groups (Kachroo et al., 2003; Truman et al., 2007). *jasmonate insensitive-1* (*jin1*) is a JA pathway mutant that has decreased sensitivity to methyl-JA (Berger, Bell and Mullet, 1996). The Kunkel lab demonstrated that *jin1* has reduced disease susceptibility to *Pst* which correlated with elevated expression of Pathogenesis-Related 1 (PR-1) that was dependent on SA accumulation (Laurie-Berry et al., 2006). Laurie-Berry et al. also demonstrated that JIN1 is required for normal *Pst* DC3000 symptom development through an SA-independent mechanism. *Pst* DC3000 produces coronatine to manipulate JIN1-dependent jasmonate signaling both to suppress SA-mediated defenses and to promote symptom development (Laurie-Berry et al., 2006). The SAR-defective *jin1* phenotype suggests that the JA signaling pathway may be important for SAR. The Cameron lab was interested to know if DIR1 was upstream or downstream of JIN1 in the SAR pathway. To investigate the position of DIR1 and JIN1 in the SAR pathway, DIR1 accumulation in the petioles of *jin1* plants was examined. If DIR1 is downstream of JIN1 then DIR1 may not be synthesized or transmitted after SAR-induction in the *jin1* mutant. Col-0 and *jin1* were inoculated with SAR-inducing *Pst* or mock-inoculated. Inoculated leaves were clipped from the plants at 17 hpi, 23 hpi and 29 hpi and allowed to exude in 1 mM EDTA for six or 15 hours as described in the previous experiment. Protein gel blot analysis revealed that wild-type SAR competent Col-0 had a DIR1 signal at all time intervals in samples induced for SAR (Figure 3, Lane 7-8) but not in mock-inoculated samples (Figure 3, Lane 10-11). Mock-inoculated *jin1* plants had no

DIR1 signal at any of the time points (Figure 3, Lane 4-5) whereas SAR-induced *jin1* leaves had a 15 kDa DIR1 signal in the 23 to 29 hpi samples (Figure 3, Lane 1) but not in the 29 to 44 hpi (Figure 3, Lane 2). The lane beside the Col-0 induced 23-29 hpi sample (Figure 3, Lane 6) is an empty lane so the band visualized here is likely spill-over. The slight band seen in the *jin1* mock-inoculated 29-44 hpi sample (Figure 3, Lane 5) is likely also spill-over as mock-inoculated *jin1* samples on other blots did not have DIR1. This suggests that JA mutant *jin1* synthesizes DIR1 and it moves down the petiole after SAR. *jin1* plants were also examined for SAR competence and did not display a SAR-defect. In a parallel experiment performed by Dr. Cameron, challenged leaves of SAR-induced *jin1* had significantly lower bacteria levels (3.2×10^5 cfu ml⁻¹) compared to mock-inoculated *jin1* (10^6 cfu ml⁻¹) (Appendix 1, Figure 19). Taken together these data suggest that JIN1 is not required for SAR.

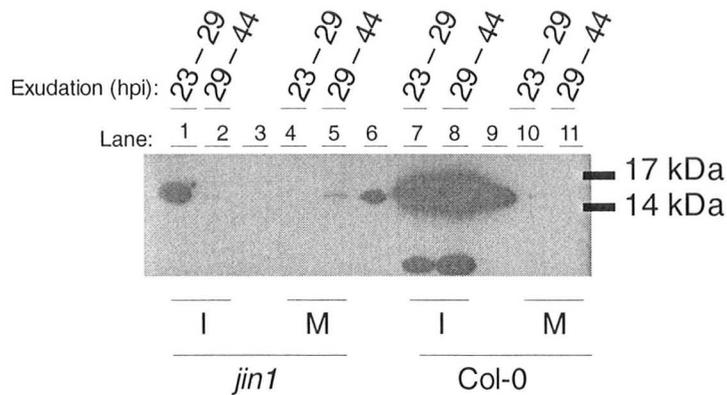


Figure 3: Anti-DIR1 protein gel blot of wild-type Col-0 and *jasmonate insensitive 1 (jin1)* petiole exudates collected 23-29 hours and 29-44 hours after SAR induction (I) or mock-inoculation (M).

2. DIR1 exists as both a monomer and dimer

One interesting observation from the DIR1 exudate protein gel blot analysis was the appearance of both ~15 kDa signal and occasionally a ~7 kDa band (Figure 2, Lane 1-3, 6-8 and Figure 3 Lane 7-8). However, DIR1 is a 77 amino acid protein with a predicted molecular weight of approximately 7 kDa (Lascombe et al., 2008). The presence of a ~15 kDa band suggests that DIR1 may exist as both a ~7 kDa monomer and a ~15 kDa dimer

in phloem-enriched petiole exudates. Protein dimers often form via non-covalent bonds. These bonds can be disrupted in the presence of reducing agents such as dithiothreitol (DTT) (Ishida et al., 1996). Many protein gel blot analysis protocols, including those used in the Cameron lab, include an incubation with DTT prior to analysis to disrupt such interactions. However tightly linked proteins many require higher than average amounts of reducing agent for dissociation to occur (Ishida et al., 1996). To investigate if the ~15 kDa DIR1 signal was due to the presence of DIR1 dimers, exudates from the DIR1 overexpressing line *dir1-1* 35S:DIR1-5E (Maldonado et al., 2002) were incubated in either 5 mM or 200 mM DTT prior to protein gel blot analysis with anti-DIR1 antibody. A DIR1 band of ~15 kDa was observed for the sample incubated in 5 mM DTT and both the ~7 kDa and ~15 kDa bands were observed for the sample incubated in 200 mM DTT (Figure 4). This suggests that the ~15 kDa DIR1 band may be a dimer of two ~7 kDa DIR1 monomers.

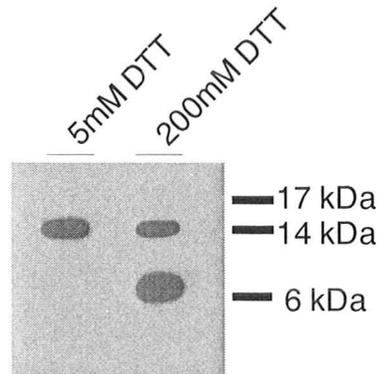


Figure 4: Anti-DIR1 protein gel blot analysis of petiole exudates (15-44 pi) from SAR-induced DIR1 overexpressing transgenic line 35S:DIR1-5E. Exudate treated with either 5mM DTT or 200mM DTT. See Appendix 1, Figure 17 for replicate experiment.

3. Use of *Agrobacterium*-mediated transient expression of DIR1 to monitor DIR1 movement

The constitutive DIR1 expression in all leaf cells of wild-type plants meant it was difficult to follow DIR1 movement by traditional visualization techniques using reporter genes. In order to observe DIR1 movement *in planta* an *Agrobacterium tumefaciens*-mediated transient expression/SAR assay was developed. This assay allows the

expression of DIR1 in one leaf of a *dir1-1* SAR-deficient plant which can then be followed during SAR, avoiding the problems of low level constitutive DIR1 expression in all leaf cells of wild-type plants. *Agrobacterium* T-DNA constructs containing either EYFP or functional DIR1 fused to EYFP under the control of the 35S promoter (35S:DIR1-EYFP) were created (Champigny et al., unpublished). *Agrobacterium* was inoculated into one lower leaf and according to Wroblewski et al. (2005) it takes four days for the T-DNA to transfer the gene of interest (EYFP or 35S:DIR-EYFP) into the plant cell and be transiently expressed. Examining EYFP localization by fluorescence microscopy failed to show a difference between mock-inoculated and *Agrobacterium*-inoculated leaves possibly due to the low expression of the construct and/or the intrinsic autofluorescence of many leaf compounds (data not shown). To verify that the construct was being expressed, reverse-transcriptase (RT)-PCR was performed on whole leaves collected four days post inoculation with *Agrobacterium*. The primers used in the RT-PCR were designed to span the junction of the DIR1-EYFP fusion, a sequence unique to the construct and not present in the native DIR1 gene. A product of the correct size was present in *dir1-1* plants infiltrated with *Agrobacterium* containing 35S:DIR-EYFP, whereas *dir1-1* plants inoculated with *Agrobacterium* containing EYFP had no such product (Figure 5). RT-PCR of mock-inoculated and untreated leaves also did not produce a DIR1-EYFP fusion product (Figure 5). This demonstrates that DIR1-EYFP was being expressed in leaves four days after *Agrobacterium* inoculation.

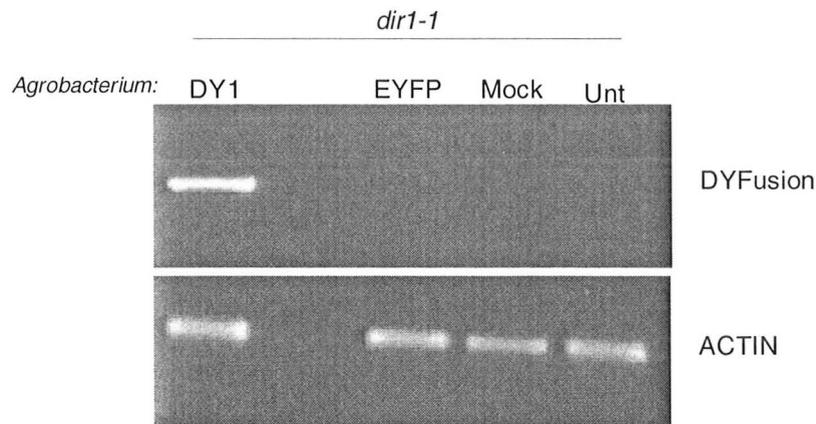


Figure 5: RT-PCR on *dir1-1* Arabidopsis leaves four days post infiltration with *Agrobacterium* carrying construct 35S:DIR1-EYFP (DY1), *Agrobacterium* EYFP, mock-inoculated with $MgCl_2$ (Mock) or Untreated (Unt). Primers DYFusion span the DIR1-EYFP construct junction with the 5' Primer in DIR1 and the 3' in EYFP. Actin was used as a loading control.

The previous data demonstrated that DIR1-EYFP was being expressed in one leaf in a *dir1-1* background. Therefore, an *Agrobacterium*-mediated transient expression was combined with the SAR assay (Agro-SAR assay). The Agro-SAR assay can be summarized as follows: one or two lower leaves per plant are inoculated with the appropriate *Agrobacterium* strain. After four days the same leaf is either induced for SAR

with *Pst(avrRpt2)* or mock-inoculated. Two days later distant leaves are challenged with virulent *Pst*, followed by determination of *Pst* levels three days post inoculation. SAR-deficient mutant lines *dir1-1* and *npr1-2* (Maldonado et al., 2002) were subjected to the Agro-SAR assay. *npr1-2* was chosen as a control plant because *NPR1* is thought to be downstream of SAR long distance signaling. Only *dir1-1* plants inoculated with *Agrobacterium* 35S:DIR1-EYFP followed by SAR-induction with *Pst(avrRpt2)* displayed reduced *Pst* levels compared to mock-inoculated plants (Figure 6). *dir1-1* plants that transiently expressed EYFP followed by mock- or SAR-inoculations supported high *Pst* levels in the challenged leaves indicating that SAR had not been established (data not shown). *npr1-2* plants supported high *Pst* levels in the challenged leaves no matter what treatment they received. These results indicate that it is possible to combine *Agrobacterium*-mediated transient expression of DIR1 with the SAR assay and it also suggests that expression of DIR1 in just one leaf followed by SAR-induction is sufficient to rescue the SAR defect in *dir1-1*. The results also suggest *NPR1* is required in distant tissues.

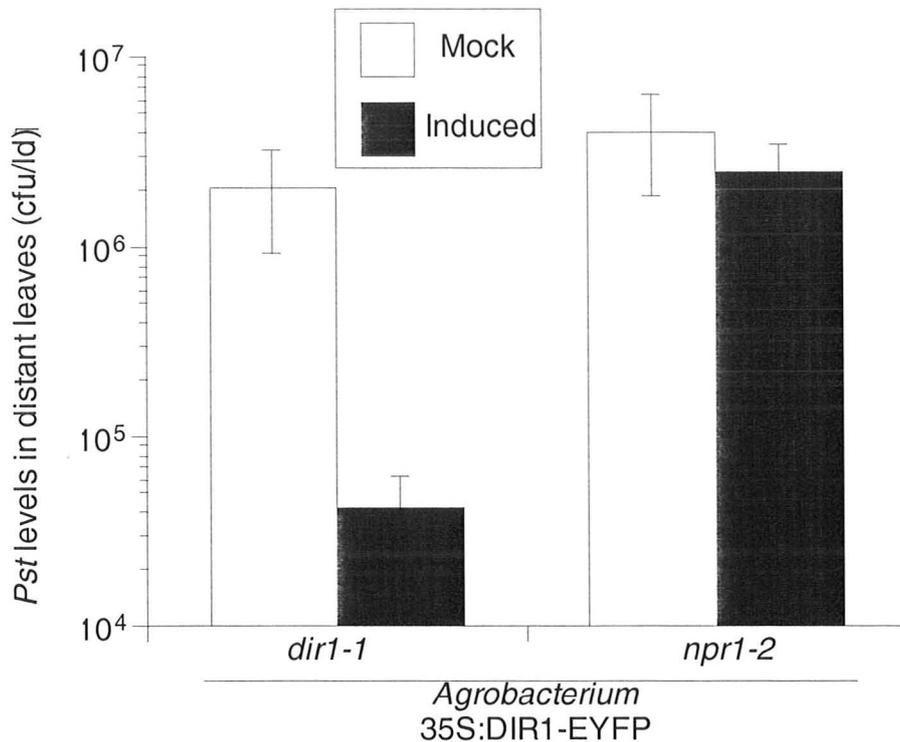


Figure 6: Bacterial levels in distant leaves of plants challenged with virulent *Pst* after all plants were inoculated with *Agrobacterium* containing 35S:DIR1-EYFP and then either SAR-induced (Induced) or mock-inoculated (Mock).

4. Use of the Agro-SAR Assay to follow DIR1 movement to distant leaves

If DIR1 is participating in the long distance signaling step of SAR, then it should move down the petiole of an induced leaf and also enter distant leaves to initiate the establishment/priming step of SAR. The Agro-SAR assay was used to investigate the movement of DIR1 to distant leaves using two SAR-defective mutants *npr1-2* and *dir1-1*. Again, *npr1-2* was chosen as a control plant because *NPR1* is thought to be required in distant tissues. *dir1-1* and *npr1-2* plants were inoculated with *Agrobacterium* containing

35S:DIR1-EYFP or EYFP followed four days later by inoculation of the same leaf with SAR-inducing *Pst(avrRpt2)* or 10mM MgCl₂ (mock-inoculation). One day after SAR-induction or mock-inoculation, exudates from distant leaves were collected from 24 to 46 hpi. Whole distant leaves were also collected at 46 hpi. Both exudates and whole leaf extracts were subjected to protein gel blot analysis using anti-DIR1 antibody. Protein concentration was determined by the Bradford assay and 48µg of each sample was loaded per lane. Neither the mock-inoculated or the SAR-induced whole leaves had detectable levels of DIR1 (Figure 7), even in undiluted samples in which protein concentrations ranged between 48µg and 176ng (Figure 7). This suggests that DIR1 is expressed via *Agrobacterium* at very low levels. Exudates collected from distant leaves that expressed either DIR1-EYFP or EYFP followed by mock-inoculation did not contain a detectable DIR1 signal, whereas those expressing DIR1-EYFP followed by SAR-induction did contain a DIR1 signal (Figure 8). Exudates collected from SAR-induced *dir1-1* expressing EYFP contained no DIR1 signal (Figure 8). The level of SAR competence was also monitored to determine if transient expression of DIR1 by *Agrobacterium* can rescue the SAR-defect in *dir1-1* or *npr1-2*. Transient expression of DIR-EYFP in *npr1-2* did not rescue the SAR defect as both SAR-induced and mock-inoculated plants had high bacterial levels. There was no reduction in SAR-induced *dir1-1* expressing EYFP compared to mock-inoculated plants. Expression of DIR1-EYFP followed by SAR-induction in *dir1-1* produced a 9-fold reduction in *Pst* levels compared to mock-

inoculated plants (Figure 9). This is a robust SAR response. DIR1 appears to also be transmitted to the distant leaves in *npr1-2* but this did not restore the SAR-defect. This suggests that DIR1 is not only moving down the petiole of leaves induced for SAR but also moving up the stem to petioles of distant leaves.

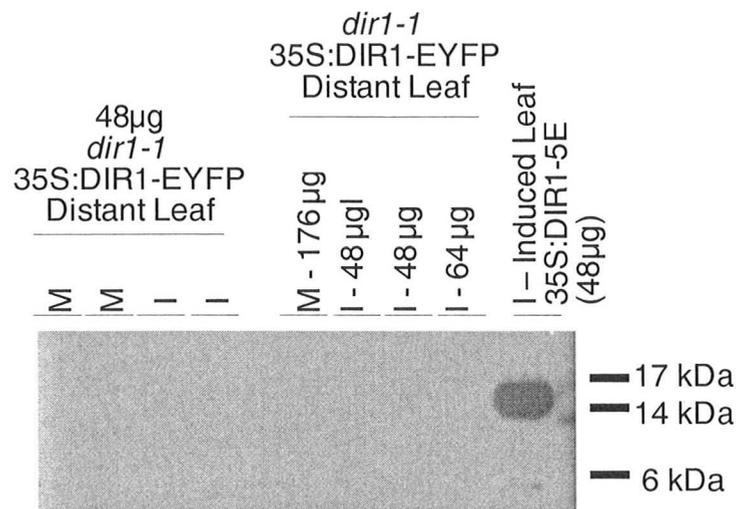


Figure 7: Anti-DIR1 protein gel blot analysis of total protein extracted from whole *dir1-1* leaves inoculated with *Agrobacterium* 35S:DIR1-EYFP and then either mock-inoculated (M) or SAR-induced (I). Petiole exudates from a SAR-induced DIR1 overexpressing line (35S:DIR1-5E) was used as a positive control.

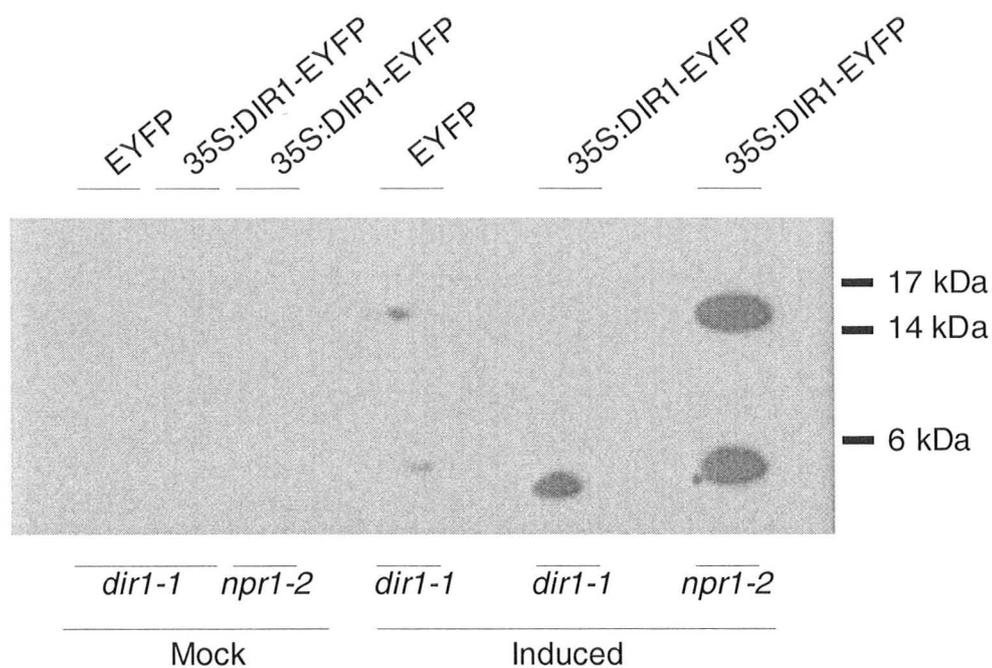


Figure 8: Anti-DIR1 protein gel blot analysis of distant leaf exudates of two SAR-defective *Arabidopsis* mutants *dir1-1* and *npr1-2*. Plants were inoculated with *Agrobacterium* containing either DIR1-EYFP or control EYFP. After *Agrobacterium* inoculation plants were either SAR-induced (Induced) or mock-inoculated (Mock). Exudates were collected 14 to 24 hpi.

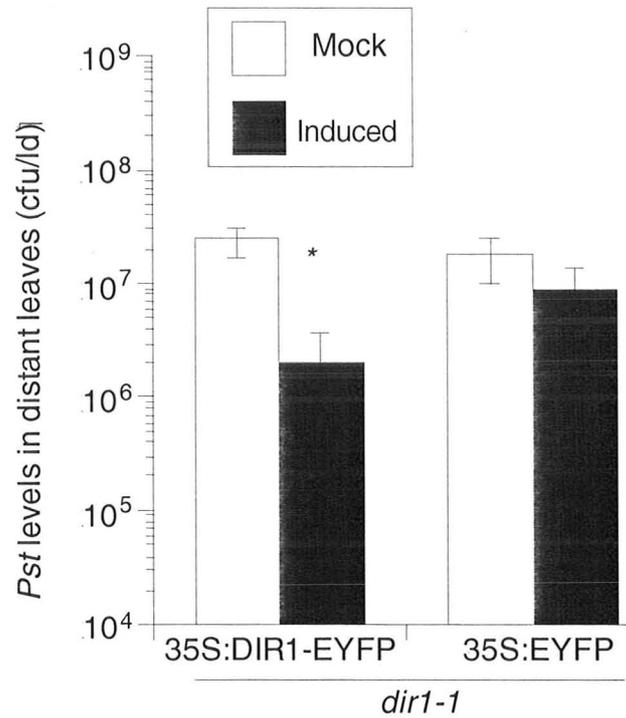


Figure 9: *In planta* bacterial levels of SAR-defective *dir1-1* after inoculation with *Agrobacterium* containing either DIR1-EYFP or EYFP, SAR-induced (black or Mock-induced (white) and challenge of distant leaves with virulent *Pst*.

5. Cucumber exudates rescue SAR-defective *dir1-1*

If DIR1 plays a central role in the SAR pathway then a DIR1-like protein should be present in other plants that exhibit SAR responses. Cucumbers have been used in a number of SAR studies and display a robust SAR response (Smith, Hammerschmidt and Fulbright, 1991). If cucumber exudates contain a protein or proteins that are biologically similar to DIR1 in Arabidopsis, then it may be possible for cucumber exudates collected from SAR-induced plants to complement the SAR defect in *dir1-1*. Cucumbers were either mock-inoculated or induced for SAR by inoculating leaves with 10^8 cfu ml⁻¹ *Pseudomonas syringae* pv *syringae* D20 (*Pss*). Based on previous work of Rasmussen et al. (1991) it was determined that the SAR signal leaves the induced leaf between four to eight hpi therefore exudates were collected starting at eight and 22 hpi using glass calibrated capillary pipets. Exudates were diluted 15-fold and infiltrated into two lower leaves of the Arabidopsis mutants *dir1-1* and *npr1-2*. As a positive control DIR1 overexpressing line 35:DIR1-5E (Maldonado *et al.*, 2002) were also infiltrated with mock-inoculated and SAR-induced cucumber exudates. Two days later distant Arabidopsis leaves were challenged with virulent *Pst* and bacterial levels were measured three days later. *npr1-2* inoculated with exudates from SAR-induced eight hpi cucumbers supported bacterial levels similar to those that received exudates from eight hpi mock-inoculated cucumber, thus *npr1-2* plants were SAR-incompetent regardless of what

cucumber exudate they received. SAR was established in *dir1-1* plants receiving exudates from SAR-induced eight hpi cucumber as demonstrated by a two-fold reduction in distant leaf *Pst* levels compared to *dir1-1* plants that received exudates from mock-inoculated cucumber. *dir1-1* plants infiltrated with exudates from 22hpi cucumber did not display SAR (data not shown). SAR was also established in DIR1 overexpressing line 35S:DIR1-5E receiving exudates from SAR-induced eight hpi cucumber exudates but not eight hpi mock-inoculated exudates or either of the 22 hpi exudates. This data suggests that exudates from eight hpi SAR-induced cucumber can rescue the SAR defect in *dir1-1* Arabidopsis and induce SAR in DIR1 overexpressing line 35:DIR1-5E and that cucumber exudates contain a long distance signal that can compensate for the absence of DIR1 in the *dir1-1* mutant.

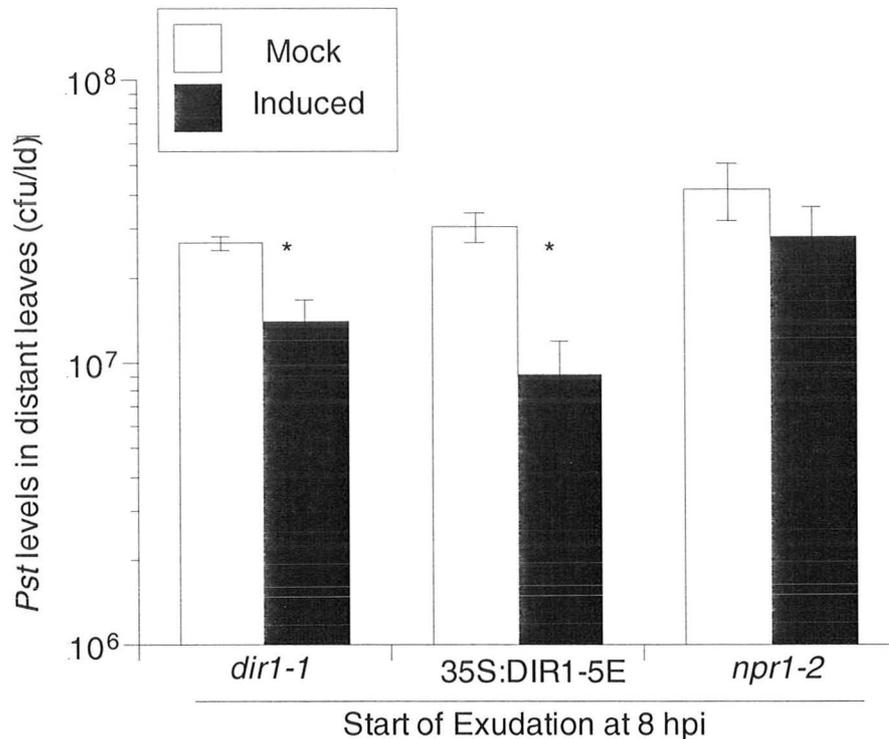


Figure 10: Bacterial levels in the distant leaf of *dir1-1*, DIR1 overexpressing line (35S:DIR1-5E) and *npr1-2*. Plants were infiltrated with exudates from a cucumber eight hours after SAR-induction. Two days later the distant leaves were challenged with virulent *Pst*. (*) show significant differences as determined by student t-test.

6. Cucumber exudates contain a DIR1-like protein

The data above demonstrates that SAR-induced cucumber exudates can rescue the SAR defect in *dir1-1* Arabidopsis. If this rescue is due to a DIR1-like protein it may be possible to detect it in the cucumber petiole exudates using the DIR1 antibody. Cucumber exudates were collected as described above and concentrated by lyophilization followed by protein gel blot analysis with the anti-DIR1 antibody. A band of approximately 15 kDa was present in all exudates at both eight and 22 hpi (Figure 11) indicating that cucumbers

express a DIR1-like protein whether they are mock-inoculated, induced for SAR or untreated.

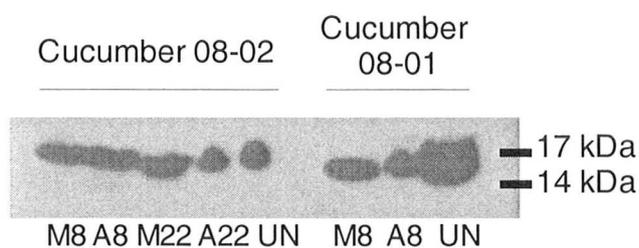


Figure 11: Anti-DIR1 protein gel blot analysis of cucumber exudates eight hours after mock-inoculation (M8), SAR-induction (A8) or 22 hours after mock-inoculation (M22) or induced for SAR (A22) or Untreated (UN). Two different experiments are shown.

Improvement of the ARR mutant Screen

1. Use of virulent *Pst*-GFPuv in ARR mutant screens

The Cameron lab is interested in elucidating the ARR pathway in Arabidopsis. One approach has been to perform genetic screens to identify potential ARR mutants. To determine if a mutagenized seed population contains a mutant in the ARR pathway, mature (six weeks post germination) plants are inoculated with 10^6 cfu ml⁻¹ virulent *Pst* DC3000 and assessed three days later for disease characteristics. Mature wild-type Arabidopsis have little to no yellowing and/or necrosis and low *in planta Pst* levels. Any plant displaying symptoms has its *in planta Pst* levels measured to confirm if its a potential ARR mutant. The current screen takes seven days to complete. During day one of the screen an overnight culture of virulent *Pst* is prepared and grown until mid-log phase (approximately 16 hours at room temperature). This culture is used on the second day to inoculate the leaves of a mature mutant population with 10^6 cfu ml⁻¹. The plants are left for three days to allow symptoms to develop. Any plant that shows symptoms has its leaves collected and assayed for *in planta* bacteria levels. The assay involves shaking leaf discs from the infected plants in silwet detergent to release the bacteria into the solution. The solution is serially diluted and plated on selective King's B media. Two days later the colonies are counted and compared to controls to determine if a strong ARR response was established in the mutant plant. A faster protocol would make it possible to screen many more plants.

Recently Wang et al. (2007) developed a transgenic line of virulent *Pst* DC3000 that constitutively expresses enhanced Green fluorescence Protein (GFPuv) carried on a stable plasmid vector. If fluorescence activity could be used to quantify the amount of *Pst* in a leaf disc instead of plating and counting colonies it would reduce the screen protocol by two days and eliminate all the materials needed to grow the *Pst* post-isolation. The researchers who developed the virulent *Pst*-GFPuv strain reported that the level of fluorescence emitted from a sample correlated with the number of bacteria present. To confirm this 5ml cultures were grown to the logarithmic growth phase (approximately 16 hours at room temperature) and diluted to varying concentrations between 10^8 cfu ml⁻¹ to 10^6 cfu ml⁻¹ based on optical density. Fluorescence and standard *Pst* quantification were performed for each dilution. Fluorescence was measured immediately in a flurometer at 485nm excitation and 535nm emission based on Wang et al. (2007). *Pst* was quantified three days post plating to confirm the level of *Pst* present in each sample (as optical density is just an estimate). Bacterial levels using the standard *Pst* isolation procedure correlated with fluorescence, only at levels above 10^7 cfu ml⁻¹ (Figure 13). Indicating there is a threshold of detection as concentrations below 10^7 cfu ml⁻¹ all fluoresced to similar levels (approximately 5 units of fluorescence) (Figure 12).

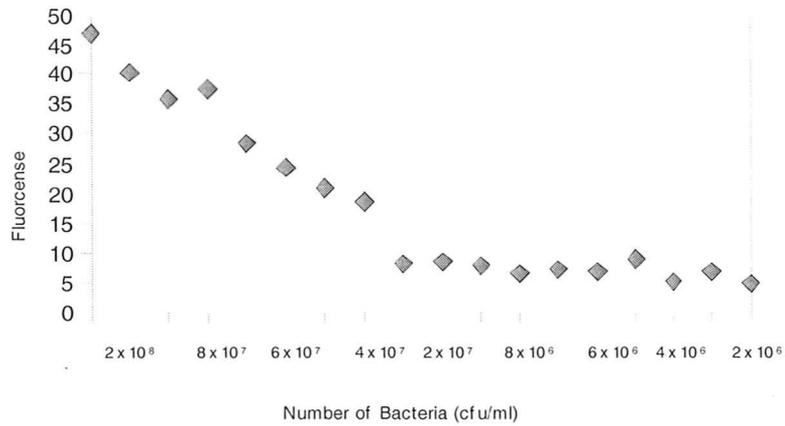


Figure 12: Concentrations of *Pst* GFPuv were estimated by optical density. A dilution series was made from 2×10^6 cfu ml⁻¹ to 9×10^7 cfu ml⁻¹ at 10-fold intervals. Fluorescence output of each sample was measured before the samples were plated using the standard *Pst* quantification measurements.

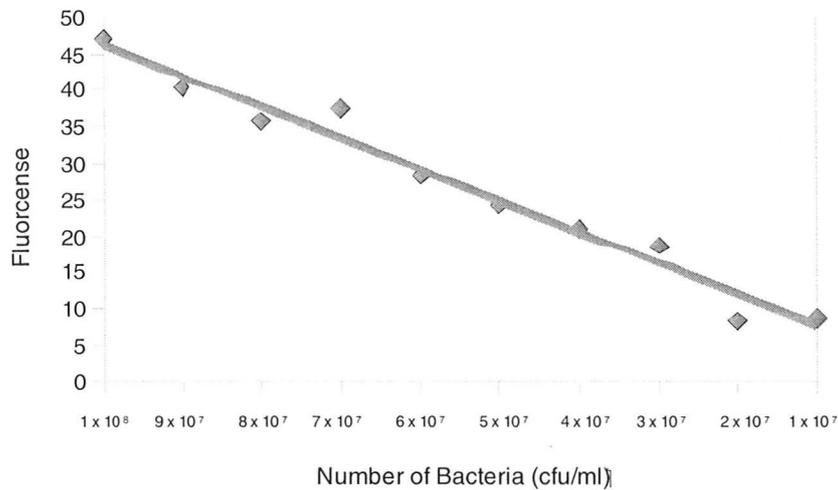


Figure 13: Part of the standard curve in the previous figure. Fluorescence of *Pst* GFPuv for concentrations between 10^8 and 10^7 cfu ml⁻¹.

2. *Pst*-GFPuv are as virulent as *Pst* DC3000 during ARR

The research team that developed the *Pst*-GFPuv strain reported that the virulence of *Pst*-GFPuv was the same as the parental strain, *Pst* DC3000, in young plants, but it was important to determine if this was also the case in mature plants during ARR. To use *Pst*-GFPuv in an ARR screen it would have to exhibit similar virulence as *Pst* DC3000. To investigate if *Pst*-GFPuv exhibited similar levels of virulence and caused similar disease symptoms, mature (six wpg) wild-type Col-0 and ARR mutants *sid2* and *iap1-1*

were inoculated with either virulent *Pst* DC3000 or with virulent *Pst*-GFPuv. After three days the disease symptoms of each was examined and bacterial levels were measured using the silwet-leaf disc and selective plating method. Col-0 leaves displayed little yellowing or lesions in plants inoculated with either virulent *Pst* or *Pst*-GFPuv (Figure 14). Both *iap1-1* and *sid2* leaves were yellowed, slightly wilted and had occasional spots of necrosis after inoculation with both *Pst* strains (Figure 14). Bacterial levels were similar in plants inoculated with *Pst* or with *Pst*-GFPuv. Col-0 exhibited a strong ARR response supporting 10^6 cfu ml⁻¹ virulent *Pst* and 9.5×10^5 cfu ml⁻¹ *Pst*-GFPuv. ARR-incompetent *iap1-1* and *sid2* displayed high *in planta* *Pst* levels (10^7 cfu ml⁻¹) for both *Pst* DC3000 and *Pst*-GFPuv. This indicates that *Pst*-GFPuv produces disease symptoms and grows to similar levels as *Pst* DC3000 in Arabidopsis during ARR.

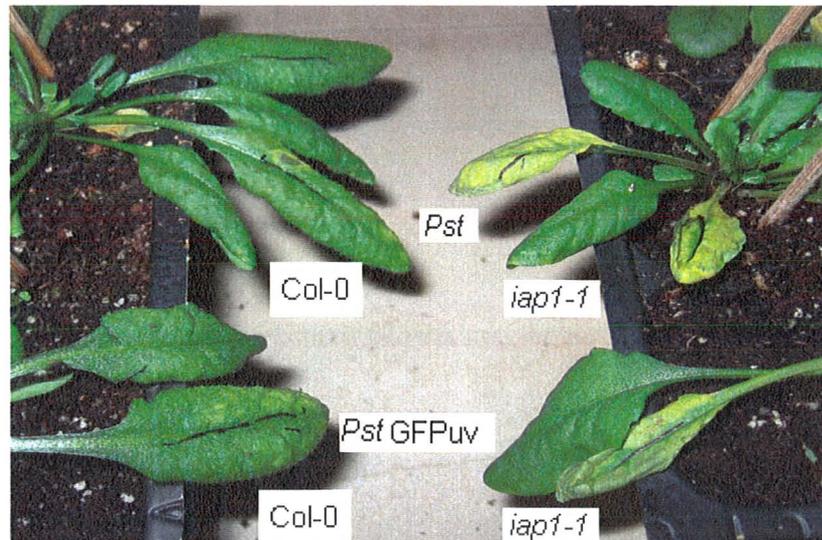


Figure 14: Photograph of Col-0 (left) and *iap1-1* (right) leaves two days after inoculation with 10^6 cfu ml⁻¹ *Pst* (top) or *Pst-GFPuv* (bottom).

3. Use of *Pst-GFPuv* to differentiate between ARR-competent wild-type and ARR-incompetent mutants

The current ARR assay as described above limits the number of plants an individual can screen in a finite time period. A labour intensive part of the screen comes from the multiple dilutions and plating steps in addition to the preparatory work required in making the selective plates. To determine if *Pst-GFPuv* plus fluorescence measurements could be used in place of the standard ARR assay, mature (6 wpg) Col-0 and the ARR mutant, *iap1-1* were inoculated with *Pst-GFPuv*. Three days later eight leaf discs were

collected from infected leaves of each ecotype, followed by shaking in silwet detergent to release the *Pst*-GFPuv into solution. After one hour of shaking 300µl of the solution was measured for fluorescence (excitation – 485nm, emission - 535nm). The solution was also plated conventionally for comparison with the fluorescence data. A clear difference was seen between the fluorescence level of the Col-0 and *iap1-1* solutions. Col-0 solution emitted an average of 100 units of fluorescence whereas the *iap1-1* solution emitted an average of 800 units of fluorescence. When compared to the previous experiment that correlated units of fluorescence to number of *Pst* this suggests that the Col-0 solution contains 10^5 cfu ml⁻¹ and *iap1-1* contains 10^8 cfu ml⁻¹. Although the difference between these two samples is clear, a problem arose when these units of fluorescence were compared to standard plating. Standard plating of the solution indicated that there were 10^6 cfu ml⁻¹ *Pst*-GFPuv present in Col-0 and 5×10^8 cfu ml⁻¹ *Pst*-GFPuv in *iap1-1*. This suggests that *Pst*-GFPuv concentration below 10^7 cfu ml⁻¹ are not distinguishable from lower concentrations (10^7 cfu ml⁻¹ is fluorescence just as much as 10^6 cfu ml⁻¹). In conclusion these experiments suggest fluorescence of *Pst*-GFPuv could be used to show relative differences between ARR competent plants and ARR mutants but the range of detection is limited.

4. Altering the level of silwet-L77 to improve the screen

Absolute fluorescence did not change with concentrations below 10^7 suggesting that there is a threshold of detection in our current methods and/or equipment. *Pst*-GFPuv concentrations below 10^7 cfu ml⁻¹ are indistinguishable from one another. Maximizing the amount of *Pst*-GFPuv released into solution could improve the sensitivity of the assay. Silwet is a detergent that interrupts the phospholipid bilayer and the connections between plant cells. It was hypothesized that increasing the concentration of silwet, may increase the fluidity of the plant membranes, loosen the plant cells and increase the number of *Pst*-GFPuv released into solution. To test this, leaf discs from *Pst*-GFPuv inoculated *iap1-1* and Col-0 were shaken in either standard 0.2% Silwet in MgCl₂ or in 2% Silwet in MgCl₂. After one hour of shaking, fluorescence of each solution was measured. There was no significant difference between samples in 0.2% Silwet and those shaken in 2% silwet. Col-0 leaf discs displayed an average of 100 units of fluorescence in 0.2% Silwet and 102 units in 2% silwet. *iap1-1* leaf discs displayed an average of 600 units of fluorescence in 0.2% and 602 units in 2% silwet. These experiments indicate that altering the concentration of Silwet detergent does not enhance or alter the amount of *Pst*-GFPuv released into solution over one hour.

5. Decreasing the number of leaf discs to improve the screen

In a continued effort to improve the ARR mutant screen another labour intensive step was considered: the time taken to collect leaf discs. Each sample requires eight leaf discs to be collected from each experimental group, decreasing this number would improve the efficiency of screening. To test if the number of leaf discs collected could be reduced, a series of eight, four, two and one leaf disc(s) were collected from Col-0 and *iap1-1* three days after inoculation with *Pst*-GFPuv. Each set of leaf discs was shaken in 1ml of 0.2% silwet detergent in MgCl₂. After one hour, fluorescence was measured. Absolute fluorescence dropped with the successive removal of leaf discs in both Col-0 and *iap1-1* however, a significant difference between them remained (Figure 12). Therefore decreasing leaf disc numbers lowers the absolute fluorescence values, but even the solution from just two leaf discs was sufficient to separate an ARR mutant from a wild-type plant.

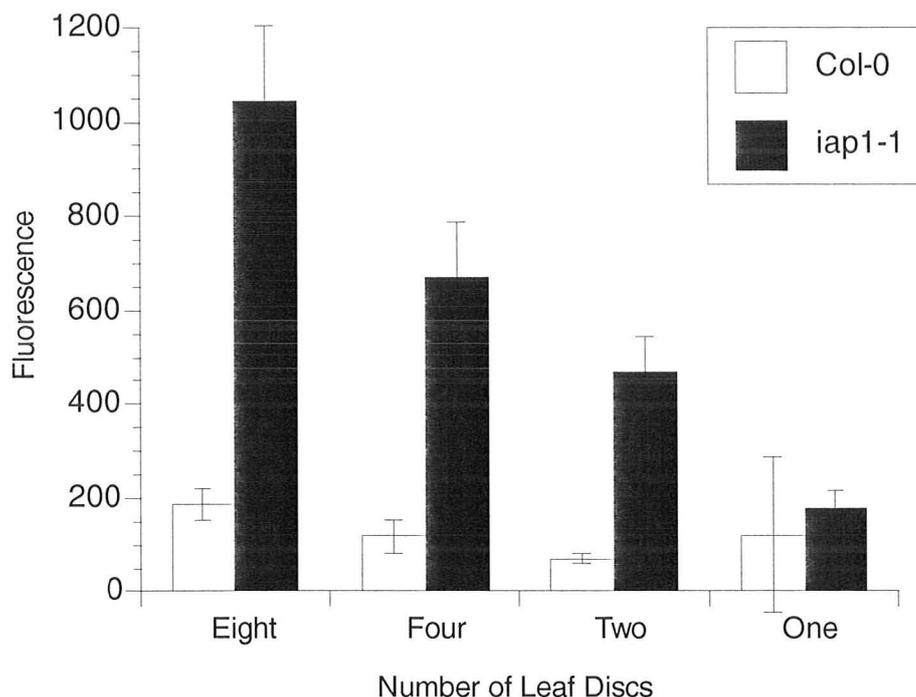


Figure 15: Wild-type Col-0 and ARR-defective *iap1-1* were inoculated with virulent *Pst* GFPuv. Three days later either eight, four, two or one leaf disc(s) were collected and shaken in 600 μ l of solution. After one hour, fluorescence of each sample was measured.

6. Decreasing the volume of silwet L-77 solution to improve the threshold of detection

As changing the concentration of silwet did not improve the efficiency of the ARR fluorescence screen it was thought that reducing the volume of the leaf disc solution could produce concentrated samples and thus increase the ability to detect *Pst*-GFPuv.

Eight leaf discs from *Pst*-GFPuv inoculated Col-0 and *iap1-1* were collected three days after inoculation and shaken in 600 μ l or 300 μ l of silwet detergent solution followed by fluorescence measurements. There was no significant difference in using 600 μ l or 300 μ l of solution (Figure 16). This suggests that the volume of leaf disc solution used can be decreased to 300 μ l, however, a reduction does not enhance the screen sensitivity.

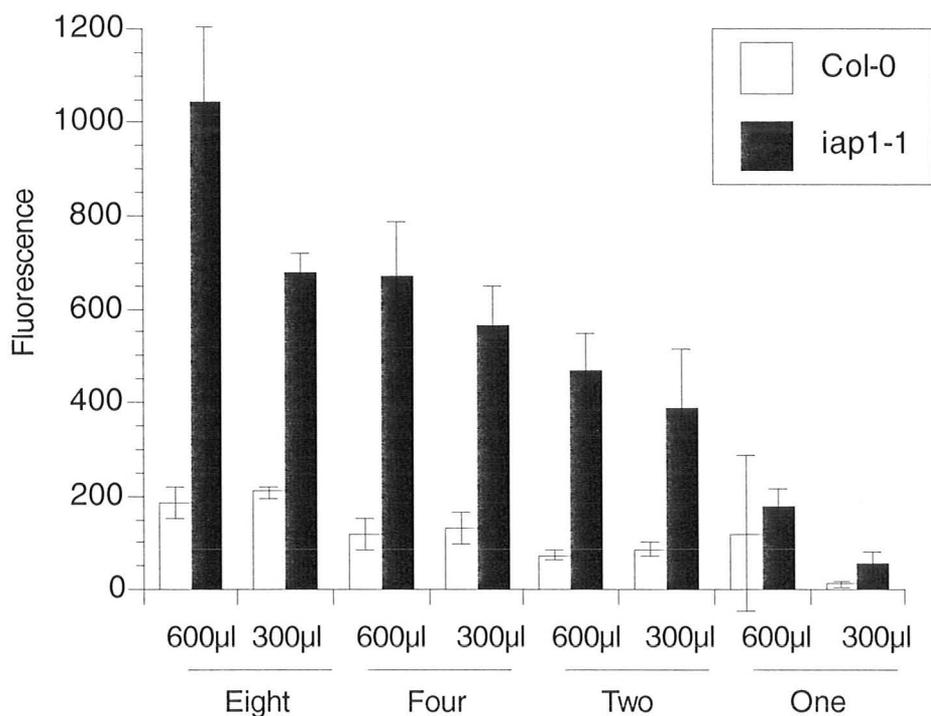


Figure 16: Leaf discs from virulent *Pst* GFPuv infected Col-0 and *iap1-1* leaf discs were shaken in either 300 μ l or 600 μ l of silwet washing solution. Fluorescence of the wash was measured. Different numbers of leaf discs in the wash were also explored (eight, four, two and one).

Discussion

DIR1 is detected in petiole exudates of Col-0 between 17 and 29 hpi

SAR-induced Col-0 had detectable levels of DIR1 in the petiole exudates at 17, 23 and 29 hpi whereas mock-inoculated Col-0 had no detectable DIR1 in the exudates at any of these time points. Not only does this demonstrate that DIR1 accumulates in the exudates in a SAR-dependent manner, it suggests that DIR1 maybe moving down the petiole such that it is detected in exudates starting at 17 hpi. DIR1 continues to be detected at 29 hpi suggesting DIR1 is still moving down the petiole. This time frame fits with the *Arabidopsis-Pst* SAR establishment window as leaves must remain on the plant for 36 to 48 hours for distant tissues to become SAR competent. It would be interesting to test if exudates after 48 hours (when the induced leaf can be removed without affecting SAR) still contained DIR1 to determine if a signal is still detectable after the SAR establishment period has passed. The absence of detectable levels of DIR1 in exudates prior to SAR induction plus the detection of DIR1 after SAR has been induced suggests that movement of DIR1 is associated with SAR induction. DIR1 may be moving down the petiole during the SAR induction stage. However, it possible that a different mobile signal travels down the petiole where it induces movement of DIR1 from cells in the petiole into the petiole exuates. Once the long distance signal has reached the distant tissues SA accumulates possibly by the conversion of methyl-SA via methyl esterase proteins such as SBP2. It is also possible that DIR1 carries methyl-SA to the distant leaf.

JIN1 is not required for SAR

Petiole exudates collected between 23 to 29 hpi from mock-inoculated *jin1* leaves did not contain a DIR1 signal, however SAR-induced *jin1* leaves contained a DIR1 signal. This suggests that DIR1 accumulation and movement into the petiole is upstream or independent of JIN1. *jin1* was SAR-competent in the Cameron lab, unlike results reported by Truman et al. (2006). Taken together these data suggest that JIN1 is not required for SAR.

Jasmonic acid been demonstrated to suppress the salicylic acid pathway. Some bacterial pathogens such as *Pseudomonas* have been demonstrated to manipulate the jasmonic acid in order to suppress SA as part of basal defense strategies. *jin1-1* mutant plants do not accumulate JA so *Pst* is not able to suppress SA-mediated resistance. As SA is present in *jin1-1* plants overall *Pst* is less successful at survival. This accounts for the nearly 10-fold reduction in *Pst* growth seen in mock-inoculated Col-0 compare to mock-inoculated *jin1-1* plants after a second inoculation challenged the distant tissues with *Pst* (Figure 18). The lack of JA-mediated SA suppression leads to SA accumulation in the induced leaf and an enhanced basal defense response.

The difference surrounding JIN1 involvement in the SAR pathway may be environmentally linked. The classic disease triangle highlights the importance that environment plays in whether or not disease is established. Discrepancy also could have come from the methods used in each. Extremely low level expression may be enough of

some molecules to elicit a response. Even though JA production is thought to be abolished in *jin1-1* plants a few molecules may still be synthesized and this may be enough to activate a response.

DIR1 exists as both a monomer and dimer

Petiole exudates from SAR-induced DIR1 overexpressing line (35S:DIR1-5E in *dir1-1*) treated with 200mM DTT contained a ~7 kDa in addition to the ~15 kDa signal. This suggests that DIR1 may exist as a 15 kDa dimer as well as a 7 kDa monomer. Covalent bonds are some of the strongest chemical interactions and contribute to protein structure. Disulfide bridges form between the sulphur atoms of two cysteine side which DIR1 has. DIR1 may form a ~14 kDa homodimer as part of the SAR signaling process.

Agro-SAR assay reveals that DIR travels to distant leaves after SAR-induction

DIR1 was detected in the exudates of distant leaves of *dir1-1* plants when DIR1-EYFP was expressed in a different leaf followed by SAR-induction. As *dir1-1* does not produce DIR1 at detectable levels in any of its leaves this suggests that transient expression DIR1-EYFP has moved from the induced leaf to the distant leaf. Traveling from induced tissues to the distant tissues is one of the key proposed SAR steps, therefore this provides additional evidence that DIR1 is an important long distance signal.

Cucumber exudates contain a SAR-inducing activity and a DIR1-like protein

Exudates collected from SAR-induced cucumber petioles were able to confer resistance to naive Arabidopsis plants including SAR-defective *dir1-1*. The fact that cucumbers contain SAR-inducing activity in Arabidopsis suggests that there is some level of conservation of the SAR pathway between the two organisms. Protein gel blot analysis of cucumber exudates with anti-DIR1 antibody revealed cucumber exudate contains a ~15 kDa signal. Untreated, SAR-induced and mock-inoculated cucumber exudates all contained this DIR1-like protein, unlike Arabidopsis in which DIR1 is detectable only in petiole exudates after SAR-induction. Cucumbers have been used in a number of SAR studies and offer a number of advantages over Arabidopsis. Cucumbers display a robust SAR response and it's possible to collect pure phloem sap unlike the 100-fold dilutions with Arabidopsis.

In the experiments that looked at DIR1 expression in tissues in Arabidopsis, GUS staining indicated that DIR1 is expressed constitutively at low levels in all cells even in mock-inoculated and untreated plants. Cucumber exudate are a more concentrated compared to Arabidopsis exudates. The DIR1-like expression seen in the mock and untreated samples maybe a result of this higher concentration. A small amount of DIR1 may be collected from Mock and Untreated Arabidopsis but because of the small quantity coupled with 100-fold dilution used during the collection process we are not able to detect these small quantities by protein immunoblotting.

The DIR1-like protein detected in the cucumber exudates may be only one of many DIR1-like proteins present in cucumbers so the biological rescue seen in *dir1-1* arabidopsis (Figure 10) may not directly correlate to DIR1 signal seen in SAR-induced, mock-inoculated and untreated cucumber exudates (Figure 11).

DIR1 by itself may not be enough to elicit a SAR response. DIR1 may have to bind a lipid for successful establishment to occur in the distant tissues.

Conclusions on SAR

DIR1 appears to be an important molecule for the long distance signaling stage of SAR. DIR1 is present in wild-type ecotype Col-0 upon SAR-induction and SAR-competent *jin1-1* plants upon SAR-induction. Transient expression of DIR1 via the newly developed SAR-Agro Assay is sufficient to rescue the SAR-defect in *dir1-1*. DIR1 is found in the petiole exudates of both the induced and distant leaves of *dir1-1* plants transiently expressing DIR1 via *Agrobacterium* inoculation suggesting DIR1 is moving via the phloem and/or cell-to-cell along the petiole. A DIR1-like molecule appears to be present in cucumbers not only suggesting DIR1 may be used in a number of species but also opening the door for Arabidopsis-cucumber experiments.

Use of *Pst* GFPuv for an ARR Screen

ARR-competent Col-0 plants infected with *Pst* GFPuv fluoresced less than ARR-deficient *iap1-1*. Fluorescence correlated with bacterial levels at higher bacterial concentrations suggesting that fluorescence could be used to identify potential ARR mutants by comparing the raw fluorescence value of a potential mutant with the raw fluorescence value of a wild-type plant. The mutant should have a significantly higher level of fluorescence.

Altering the volume of silwet improves the screen but changing the concentration does not

As there seemed to be a threshold to the fluorescence of *Pst* GFPuv at concentrations below 10^7 cfu ml⁻¹ a number of attempts were made to improve the screen. The ARR screen involves shaking leaf discs in a detergent solution release the bacteria from the intercellular space. Col-0 leaf discs displayed an average of 100 units of fluorescence in 0.2% Silwet and 102 units in 2% silwet. *iap1-1* leaf discs displayed an average of 600 units of fluorescence in 0.2% and 602 units in 2% silwet. These experiments indicate that altering the concentration of Silwet detergent does not enhance or alter the amount of *Pst*-GFPuv released into solution over one hour.

Altering the number of leaf discs improves the screen

To develop the screen further it would be interesting to see if the difference seen in the fluorescence of *iap1-1* and Col-0 is actually due to some pathogen-plant specific interaction. To test this use a different ARR mutant other than *iap1-1* could be used such as *sid2*.

Conclusions on ARR

In conclusion future work can center around shaking two leaf discs per plant in 300µl of solution. It may be possible to use a lower volume of solution as volumes less than 300µl were not explored. An interesting next step would be to perform the screen on a mutant population and see if a strong ARR mutant can successfully be detected.

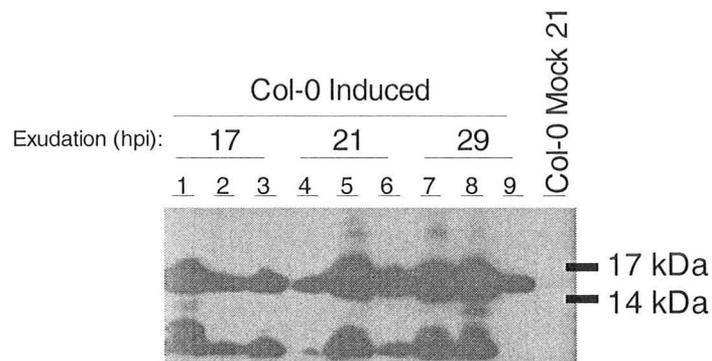
Appendix A – Supplementary Figures

Figure 17: Anti-DIR1 protein gel blot of petiole exudates from SAR-induced and mock-inoculated Col-0. Petioles were removed and exuded for various lengths of time starting at 17-23, 23-29 and 29-44 hpi.

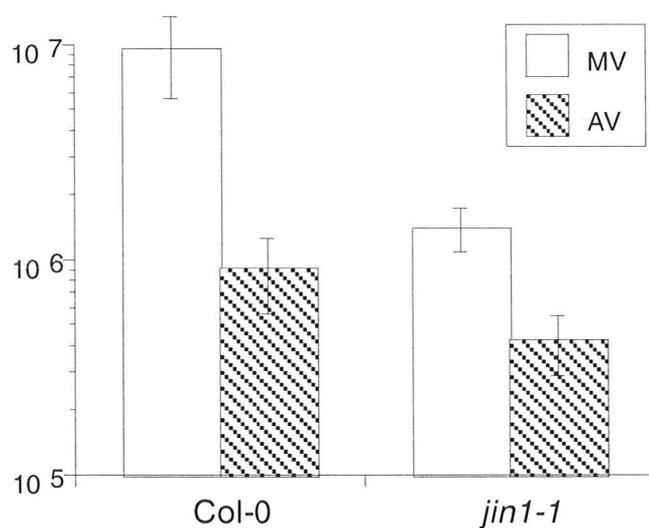


Figure 18: SAR Assay of Col-0 and *jin1-1*. Plants were either mock-inoculated with MgCl₂ (M) or induced for SAR with *Pst* (*avrRpt2*) (A) on two or three lower leaves. Three days later upper leaves were challenged with virulent *Pst* (V). Bacterial levels in the distant leaves were quantified. This experiment was performed in parallel with collection of *jin1-1* petiole exudates (Figure 3). This experiment was performed by Dr. Cameron.

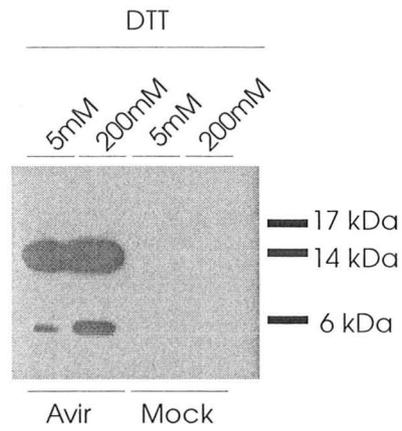


Figure 20: Anti-DIR1 protein gel blot analysis of petiole exudates (15 - 44 hpi) from SAR-induced DIR1 overexpressing transgenic line 35S:DIR1-5E. Exudate was treated with either 5mM DTT or 200mM DTT.

Reference

Attaran, E., Zeier, T., Griebel, T. and Zeier, J. (2009) Methyl Salicylate Production and Jasmonate Signaling are not essential for Systemic Acquired Resistance in *Arabidopsis*. *Plant Cell* 21, 954-971.

Bowling, S., Clarke, J., Lui, Y., Klessig, D. and Dong, X. (1997) The *cpr5* mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell* 9(9), 1573-1584.

Bradford, M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemistry* 72, 284-254.

Burger, S., Bell, E. and Mullet, J. (1996) Two methyl jasmonate-insensitive mutants show altered expression of *AtVsp* in response to methyl jasmonate and wounding. *Plant Physiology* 111, 525-531.

Cameron, R., Dixon, R. and Lamb, C. (1994) Biologically Induced Systemic Acquired Resistance in *Arabidopsis thaliana* *Plant Journal* 5(5), 715-725.

Cameron, R., Paiva, N., Lamb, C. and Dixon, R. (1999) Accumulation of salicylic acid and PR-1 gene transcripts in relation to the systemic acquired resistance (SAR) response induced by *Pseudomonas syringae* pv. *tomato* in *Arabidopsis*. *Physiological and Molecular Plant Pathology* 55, 121-130.

Cameron, R. and Zaton, K. (2005) Intercellular salicylic acid accumulation is important for age-related resistance in *Arabidopsis* to *Pseudomonas syringae*. *Physiological and Molecular Plant Pathology* 65, 197-209.

Carviel, J., Al-Daoud, F., Neumann, M., Mohammad, A., Provart, N., Moedere, W., Yoshioka, K. and Cameron, R. (2009) Forward and reverse genetics to identify genes involved in the age-related resistance response in *Arabidopsis thaliana*. *Molecular Plant Pathology* 10 (5), 621-634.

Champigny, M. and Cameron, R. (Unpublished) Action at a distance: long distance signals in induced resistance.

Champigny, M., Shearer, H., Faubert, J., Melsa, M., Mohammad, A., Haines, K., Neumann, M., Yang He, S., Fobert, P., Dengler, N. and Cameron, R. (Unpublished)

Localization of DIR1, a putative Lipid Transfer Protein involved in long distance signaling during Systemic Acquired Resistance.

Chaturvedi, R., Krothapalli, K., Makandar, R., Nandi, A., Sparks, A., Roth, M., Welti, R. and Shah, J. (2008) Plastid w³-fatty acid desaturase-dependent accumulation of a systemic acquired resistance activity in petiole exudates of *Arabidopsis thaliana* is independent of jasmonic acid. *The Plant Journal* 54, 106-117.

Cui, H., Xiang, T. and Zhou, J. (2009) Plant Immunity: a lesson from pathogenic bacterial effector proteins. *Cellular Microbiology* 11(10), 1453-1461.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weyman, K., Negrotto, D., Gaffney, T., Gut-Rela, M., Kessman, H., Ward, E. and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. *Science* 266, 1247-1250.

Dewdney, J., Reuber, T., Wildermuth, M., Devoto, A., Cui, J., Stutius, L., Drummond, E. and Ausubel, F. (2000) Three unique mutants of *Arabidopsis* identify eds loci required for limiting growth of a biotrophic fungal pathogen. *Plant Journal* 24(2), 205-218.

Eckardt, N. and Innes, R. (2003) Resistance rodeo: rounding up the full complement of *Arabidopsis* NBS-LRR genes. *Plant Cell* 15, 806-807.

Friedrich, L., Vernooij, B., Gaffney, T., Morse, A. and Ryals, J. (1995) Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. *Plant Molecular Biology* 29, 959-968.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann H. and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 261, 754-756.

Guedes, M.E.M., Richmond, S. and Kuc, J. (1980). Induced systemic resistance to anthracnose in cucumber as influenced by the location of the inducer inoculation with *Colletotrichum lagenarium* and the onset of flowering and fruiting. *Physiological Plant Pathology* 17, 229-233.

Hammerschmidt, R., Acres, S. and Kuc, J. (1976) Protection of Cucumber against *Colletotrichum-lagenarium* and *Colletotrichum-cucumberinum*. *Phytopathology* 66, 790-793.

Hugot, K., Amie, S., Conrod, S., Poupet, A. and Galiana, E. (1999) Developmental regulated mechanisms affect the ability of a fungal pathogen to infect and colonize tobacco leaves. *Plant Journal* 20, 163-170.

Ishida, T., Narita, M., Nozaki, M. and Horiike, K. (1996). Selective cleavage and modification of the intersubunit disulfide bonds of bovine dopamine beta-monooxygenase: Conversion of tetramer to active dimer. *Journal of Biochemistry* 120 (2), 346-352.

Jenns, A.E. and Kuc, J. (1979). Graft transmission of systemic resistance of cucumber to anthracnose induced by *Colletotrichum lagenarium* and tobacco necrosis virus. *Phytopathology* 69, 753-756.

Jia, Y., McAdams, S., Bryan, G., Hershey, H. and Valent, B. (2000) Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO Journal* 19, 4004-4014.

Jones, J. and Dangl, J. (2006) The plant immune system. *Nature* 444, 323 – 329.

Jung, H., Tschaplinski, T., Wang, L., Glazebrook, J. and Greenberg, J. (2009) Priming in Systemic Plant Immunity. *Science* 324, 89-91.

Kachroo, A., Lapchyk, L., Fukushige, H., Hildebrandd, D., Klessig, D. and Kachroo, P. (2003). Plastidial Fatty Acid Signaling Modulates Salicylic Acid- and Jasmonic Acid-Mediated Defense Pathways in the *Arabidopsis ssi2* mutant. *The Plant Cell* 15, 2952-2965.

Kiba, A., Tomiyama, H., Takahashi, H., Hamada, H., Ohnishi, K., Okuno, T. and Hikichi, Y. (2003) Induction of resistance and expression of defense-related genes in tobacco leaves infiltrated with *Ralstonia solanacearum*. *Plant and Cell Physiology* 44, 287-295.

Kiefer, I.W. and Slusarenko, A.J. (2003). The pattern of systemic acquired resistance induction within the *Arabidopsis* rosette in relation to the pattern of translocation. (2003). *Plant Physiol.* 132, 840-847.

Kim, M., Kim, S., Kim, W., Mackey, D. and Lee, S. (2008) Responses of *Arabidopsis thaliana* to Challenge by *Pseudomonas syringae*. *Molecular Cells* 25 (3), 323 – 331.

Kim, Y., Hwang, B. and Park, K. (1989) Expression of Age-related Resistance in pepper plants infected with *Phytophthora sojae*. *Plant Disease* 73, 745-747.

Kuc, J. (1982). Induced immunity to plant disease. *Bioscience* 32, 854-856.

Kumar, D., Park, S., Forouhar, F., Yang, Y., Vlot, A., Fridman, E., Chaing, Y., Acton, T., Shulaev, V., Montelione, G., Pichersky, E., Tong, L. and Klessig, D. (2005) Identification of SA-binding protein 2 (SABP2) as a critical component of plant innate immunity. *Phytopathology* 95, S55-S55.

Kus, J., Zaton, K., Sarkar, R. and Cameron, R. (2002) Age-Related Resistance in *Arabidopsis* is a Developmentally Regulated Defense Response to *Pseudomonas syringae*. *The Plant Cell* 14, 479-490.

Lascombe, M., Bakan, B., Buhot, N., Marion, D., Blein, J., Larue, V., Lamb, C. and Prange, T. (2008) The structure of 'defective in induced resistance' protein of *Arabidopsis thaliana*, DIR1, reveals a new type of lipid transfer protein. *Protein Science* 17, 1522-1530.

Laurie-Berry, N., Joardar, V., Street, I. and Kunkel, B. (2006). The *Arabidopsis thaliana* JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic acid-dependent defense during infection by *Pseudomonas syringae*. *Molecular Plant-Microbe Interactions* 19, 789-800.

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S. and Ryals, J. (1995). Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. *Mol. Plant-Microbe Interact.* 8, 863-870.

Leisner, S., Turgeon, R. and Howell, S. (1993) Effects of Host Plant Development and Genetic-Determination on the Long-Distance Movement of Califlower Mosaic Virus in *Arabidopsis*. *Plant Cell* 5, 191 – 202.

Lindeberg, M., Myers, C. Collmer, A. and Schneider, D. (2008) Roadmap to New Virulence Determinants in *Pseudomonas syringae*: Insights from Comparative Genomics and Genome Organization. *MPMI* 21 (6), 685-700.

Mackey, D., Holt, B., Wigg, A. and Dangl, J. (2002) RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in *Arabidopsis*. *Cell* 108, 743-754.

Malamy, J., Carr, J.P., Klessig, D.F. and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* 250, 1002-1004.

Maldonado, A., Doerner, P., Dixon, R., Lamb, C. and Cameron, R. (2002). A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. *Nature* 419, 399-403.

Mettraux, J-P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gandin, J., Raschforf, K., Schmid, E., Blum, W. and Inverardi, B. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250 1004-1006.

Meyers, B., Kozik, A., Griego, A., Kuang, H. and Michelmore, R. (2003) Genome-wide analysis of NBS-LRR encoding genes in *Arabidopsis*. *Plant Cell* 15, 809-834.

Miller, M. (1983) Relationship between onion leaf age and susceptibility to *Alternaria porri*. *Plant Disease* 67, 284-286.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiology Plant* 15(3), 473-497.

Nandi, A., Welti, R. and Shah, J. (2004). The *Arabidopsis thaliana* dihydroxyacetone phosphate reductase gene SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1 is required for glycerolipid metabolism and for the activation of systemic acquired resistance. *Plant Cell* 16, 465-477.

Nawrath, C., Heck, S., Parinthewong, N. and Mettraux, J. (2002) EDS5, an essential component of salicylic acid-dependent signalling for disease resistance in *Arabidopsis*, is a member of the MATE transporter family. *Plant Cell* 14(1), 275-286.

Nurnberger, T., Brunner, F., Kemmerling, B. and Piater, L. (2004) Innate immunity in plants and animals: striking similarity and obvious difference. *Immunological Reviews* 198, 249-266.

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

- Pallas, J., Paiva, N., Lamb, C. and Dixon, R. (1996) Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. *Plant Journal* 10, 281-293.
- Park, S., Kaimoyo, E., Kumar, D., Mosher, S. and Klessig, D. (2007) Methyl salicylate is a critical mobile signal for plant Systemic Acquired Resistance. *Science* 5, 113-116.
- Pozo, M., Van Loon, L. and Pieterse, C. (2005). Jasmonates – Signals in Plant-Microbe Interactions. *Journal of Plant Growth Regulation* 23, 211-222.
- Rasmussen, J.B., Hammerschmidt, R., and Zook, M.N. (1991). Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae*. *Plant Physiol* 97, 1342-1347.
- Rusterucci, C., Zhao, Z., Haines, K., Mellersh, D., Neumann, D. and Cameron, R. (2005) Age-related resistance to *Pseudomonas syringae* pv. *tomato* is associated with the transition to flowering in *Arabidopsis* and is effective against *Peronospora parasitica*. *Physiological and Molecular Plant Pathology* 66, 222 – 231.
- Smith, J.A., Hammerschmidt, R. and Fullbright, D.W. (1991). Rapid induction of systemic resistance in cucumber by *Pseudomonas syringae* pv *syringae*. *Physiol. Mol. Plant Pathol.* 33, 255-261.
- Staskawicz, B., Ausubel, F., Baker, B., Elles, J. and Jones, J. (1995) Molecular-Genetics of Plant-Disease Resistance. *Science* 268, 661-667.
- Stintzi, A. and Browse, J. (2000) The *Arabidopsis* male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. *PNAS* 97, 10625-10630.
- Strobel, N., Ji, C., Gopalan, S., Kuc, J. and He, S. (1996) Induction of Systemic Acquired Resistance in cucumbers by *Pseudomonas syringae* pv *syringae* 61 HrpZ(Pss) protein. *Plant Journal* 9(4), 431-439.
- Sunderwirth, S. and Roelfs, A. (1980) Greenhouse evaluation of the adult-plant resistance to SR2 to wheat stem rust. *Phytopathology* 70, 634-637.
- Theologis, A., Ecker, J., Palm, C., Federspiel, N. and Davis, R. (2000) Sequence and analysis of the plant *Arabidopsis thaliana*. *Nature*. 408, 816-820.

Truman, W., Bennett, M., Kubigsteltig, I., Turnbull, C. and Grant, M. (2007) *Arabidopsis* systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. PNAS 104, 1075-1080.

Tuzun, S. and Kuc, J. (1985). Movement of a factor in tobacco infected with *Peronospora tabacina* Adam which systemically protects against blue mold. *Physiological Plant Pathology* 26, 321-330.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A. Ward, E. and Ryals, J. (1992). Acquired resistance in *Arabidopsis*. *Plant Cell* 4, 645-656.

Uknes, S., Winter, A.M., Delaney, T., Vernooij, B., Morse, A., Friedrich, L., Nye, G., Potter, S., Ward, E. and Ryals, J. (1993). Biological induction of systemic acquired resistance in *Arabidopsis*. *Mol. Plant-Microbe Interact.* 6, 692-698.

van Loon, L.C. and van Strien, E.A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.* 55, 85-97.

Wang, K., Kang, L., Anand, A., Lazarovits, G. and Mysore, K. (2007) Monitoring in planta bacterial infection at both cellular and whole-plant levels using the green fluorescent protein variant GFPuv. *New Phytologist* 174, 212-223.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Widerhold, D.L., Alexander, D.C., Ahl-Goy, P., Mettraux, J.P. and Ryals, J. (1991). Co-ordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3, 49-59.

Whalen, M.C., Innes, R.W., Bent, A.F. and Staskawicz, B.J. (1991). Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* 3, 49-59.

Wilcockson, J. and Hull, R. (1974) The rapid isolation of plant virus RNAs using sodium perchlorate. *Journal of General Virology* 23, 107-111.

Wildermuth, M., Dewdney, J., Wu, G. and Ausubel, F. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562-565.

Wolfe, J., Hutcheon, C., Higgins, V. and Cameron, R. (2000) A functional gene-for-gene interaction is required for the production of an oxidative burst in response to infection with avirulent *Pseudomonas syringae* pv. tomato in *Arabidopsis thaliana*. *Physiological and Molecular Plant Pathology* 56, 253-261.

Wyatt, S., Pan, S. and Kuc, J. (1991) Beta-1,3-Glucanase, Chitinase, and Peroxidase-activity in tobacco tissue resistance and susceptible to Blue Mold as related to flowering, age and sucker development. *Physiological and Molecular Plant Pathology* 39, 433-440.

Yalpani, N., Silverman, P., Wilson, T.M.A., Kleier, D.A. and Raskin, I. (1991). Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. *Plant Cell* 3, 809-818.

Yalpani, N., Shulaev, V. and Raskin, I. (1993) Endogenous Salicylic-Acid Levels Correlate to Accumulation of Pathogenesis-Related Proteins and Virus-Resistance in Tobacco. *Phytopathology* 83, 702-708.

Zhou, N., Tootle, T., Tsui, F., Klessig, D. and Glazebrook, J. (1998) PAD4 functions upstream from salicylic acid to control defense response in *Arabidopsis*. *Plant Cell* 10, 1021-1030.