IAP1'S ROLE IN ARR AND OTHER DISEASE RESISTANCE PATHWAYS

ELUCIDATION OF *IAP1*'S ROLE IN AGE-RELATED RESISTANCE AND OTHER DISEASE RESISTANCE PATHWAYS IN *ARABIDOPSIS*

By

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Abstract

Age-Related Resistance (ARR) has been observed in numerous plant species, resulting in increased disease resistance as the plant matures. The ARR defective mutant, *iap1-1*, (important in the ARR pathway,) was discovered in an ARR mutant screen and *EDS1*, (enhanced disease susceptibility,) which is involved in other disease resistance pathways, was shown to be required for ARR. Intercellular accumulation of salicylic acid (SA) is required for ARR suggesting that SA may act as an anti-microbial agent. Mature (6 wpg) *iap1-1* does not accumulate intercellular or intracellular SA in response to *Pst* inoculation. Intercellular and intracellular SA accumulation is also partially reduced in young (4 wpg) plants during R gene-mediated resistance to *Pst(AvrRpt2)* which is partially compromised suggesting that the two pathways share common elements. The novel discovery of the presence of intercellular SA during R gene-mediated resistance suggests that it may act as an anti-microbial agent during R gene-mediated resistance as it is hypothesized to during ARR. The *iap1-1* mutation maps to chromosome four between 17,938,268bp and 18,133,423b. The semi-dominant, loss of function nature of the *iap1-1* mutation suggests that *IAP1* is a positive regulator in the ARR pathway.

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Table of Contents

Abstract iii

Acknowledgments iv

List of Figures vii

List of Tables viii

Abbreviations ix

Chapter 1: Introduction

Basal resistance 1
R gene-mediated resistance 2
Systemic acquired resistance 7
Induced systemic resistance 7
The wound response 9
Age-related resistance 12
The role of salicylic acid 14
Elucidating the ARR pathway 19
Research Objectives 21

Chapter 2: Methods

Plant Growth Conditions 24
Pst Disease Resistance Assays 24
Positional mapping of *iap1-1* 28
ARR screen using florescent *Pst* 28
SA infiltration experiments, intercellular washing fluid (IWFs) collection and SA determination 32
Analysis of ARR-related gene expression using RT-PCR 34
Measuring HR 35

Chapter 3: Are other defense response genes required for ARR

eds1-1 is ARR defective 37
The ARR response occurs in *jar1-1* and *jin1-1* 39
The ARR response occurs in Cytochrome P450 (CYP71A13) T-DNA insertion lines 42
The ARR response occurs in RLK T-DNA insertion lines 44
Disease resistance in *iap1-1* 47

Chapter 4: The role of *IAP1* during ARR, Basal Resistance, and R gene-mediated resistance

SA infiltration partially rescues the *iap1-1* ARR defect..... 49
SA infiltration does not rescue the *eds1-1* ARR defect..... 52
Measure of anti-microbial activity in intercellular washing fluids in *iap1-1* compared to Col-0.....54
SA accumulation is reduced in mature *iap1-1* in response to *Pst*.....57
iap1-1 is not compromised in basal resistance..... 60
*iap1-1*displays SAR.....62
iap1-1 is partially compromised in R gene-mediated resistance to *Pst(avrRpt2)* 64
iap1-1 is partially compromised in R gene-mediated resistance to *Pst(avrRps4)*..... 66
iap1-1 mutants display a less robust HR compared with wild type..... 69
ARR gene expression analysis in Col-0 and *iap1-1*..... 72
Intercellular SA accumulation is reduced in young *iap1-1* plants in response to *Pst(avrRpt2)* 75
Intracellular SA accumulation is reduced in young *iap1-1*plants in response to *Pst* and *Pst(AvrRpt2)* 78

Chapter 5: Map-based cloning to identify *IAP1*..... 81

Chapter 6: Discussion.....92

Appendix A – Replicate Experiments. 105

References.....133

List of Figures

Figure 1 The zig zag model illustrates the interaction of the plant immune system with virulent pathogens....7
Figure 2 JA is required for the wound response pathway and induced systemic resistance....11
Figure 3 Multiple SA biosynthesis pathways in *Arabidopsis*....8
Figure 4 ARR model based on previous SA rescue experiments and the ARR microarray....23
Figure 5 The diffusion method of measuring bacterial density versus traditional grinding methods....27
Figure 6 Use of fluorescent *Pst* to quantify bacterial growth....31
Figure 7 *eds1-1* is deficient for ARR....38
Figure 8 *jar1-1* and *jin1-1* display ARR....41
Figure 9 CYP71A13 T-DNA insertion plants display ARR....43
Figure 10 RLK is up-regulated during ARR and RLK T-DNA insertion lines (SALK 119133 and SALK 126918) display ARR....46
Figure 11 Disease resistance in *iap1-1*....48
Figure 12 SA application in *iap1-1* and Col-0....51
Figure 13 SA application in *eds1-1* and Col-0....53
Figure 14 Anti-microbial activity in intercellular washing fluids collected from ARR-induced leaves....56
Figure 15 Intercellular and intracellular SA in mature (6wpg) *iap1-1* and Col-0....59
Figure 16 Basal resistance in *iap1-1*....61
Figure 17 SAR in the *iap1-1*....63
Figure 18 R gene-mediated resistance to *Pst(avrRpt2)*in *iap1-1*....65
Figure 19 R gene-mediated resistance to *Pst(avrRps4)* in *iap1-1*....68
Figure 20 HR in *iap1-1* and Col-0....71
Figure 21 ARR gene expression analysis in *iap1-1* and Col-0....74
Figure 22 Intercellular SA in young (3wpg) *iap1-1* and Col-0....77
Figure 23 Intracellular SA in young (3wpg) *iap1-1* and Col-0....80
Figure 24 *In planta* bacterial density in T-DNA insertion lines, *iap1-1* and Col-0....86
Figure 25 *IAP1* candidate gene expression analysis in *iap1-1* and Col-0...88
Figure 26 Complementation in *iap1-1*....91
Figure 27 ARR model based on previous SA rescue experiments, the ARR microarray, and expression of ARR genes in *iap1-1*....101

List of Tables

Table 1: Primers used in RT-PCR experiment 35

Abbreviations

ARR	Age-Related Resistance
avr	avirulence gene
cfu	colony forming unit
COI1	coronatine insensitive
Col-0	Columbia ecotype of *Arabidopsis*
DNA	deoxyribonucleic acid
dpg	days post germination
dpi	days post inoculation
EDS1	enhanced disease susceptibility
ETI	effecter triggered immunity
ETS	effecter triggered susceptibility
GFP	green florescent protein
HR	hypersensitive response
IAP1	important for the ARR pathway
IWFs	intercellular washing fluids
JA	jasmonic acid
JAR1	jasmonate-resistant
JIN1	jasmonate insensitive
KB	King's B media
LB	Luria broth media
ld	leaf disc
Ler	*Landsberg erecta* ecotype
MAMP	microbe associated molecular pattern
$MgCl_2$	magnesium chloride
NDR1	nonrace-specific disease resistance
NPR1	non-expresser of PR-1
OD	optical density
PAD4	phytoalexin deficient
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction
PR	pathogenesis related
Pst	*Pseudomonas syringae pv. Tomato*
PTI	PAMP triggered immunity
R gene	resistance gene
RLK	receptor-like kinase
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SA	salicylic acid
SAG	salicylic acid β-glucoside;
SAG101	senescence associated gene
SAR	systemic acquired resistance
T-DNA	transfer-deoxyribonucleic acid

UTR	untranslated region
WS	Wassilewskija ecotype of *Arabidopsis*
WPG	weeks post germination

Chapter 1: Introduction

The interaction of plants and pathogens has led to the evolution of many complex defense pathways (Maor and Shirasu, 2005). The goal of this project was to contribute to the elucidation of one of these pathways, specifically the Age-Related Resistance (ARR) pathway in *Arabidopsis*. Knowledge of the ARR pathway could be used to increase disease resistance in crop plants.

Basal resistance

An initial line of defense encountered by pathogens is referred to as basal resistance in which recognition of conserved microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs) such as bacterial flagellin leads to basal defense induction (Heath, 2000; Eulgem, 2005). Defense mechanisms employed by the plant in response to the detection of MAMPS or PAMPs is sometimes referred to as PAMP triggered immunity (PTI) and includes callose deposition that reinforces the cell wall (Kim et al., 2005), the production of reactive oxygen species (ROS) (Nurnberger et al., 2004), and salicylic acid (SA)-dependent expression of defense related genes including PR-1 (pathogenesis related) (Navarro et al., 2004; Ham et al., 2007). For example, *Arabidopsis* treated with the MAMP flg22 (bacterial flagellin) accumulate SA within six hours of treatment and expression profiling revealed the up-regulation of many defense genes (Tsuda et al., 2008). PTI can also be observed when plants are inoculated with a non-host such as *Pseudomonas syringae pv*

phaseolicola which induces both PR-1 accumulation and two types of callose deposition (Ham et al., 2007).

Some pathogens have evolved the ability to suppress or elude plant basal resistance. For example, certain pathogens, including those in the *Pseudomonas* genus, have evolved virulence effectors, which are delivered to the plant cell using a type-three secretion system. Some effectors inhibit MAMP-triggered basal defenses (Hauck et al., 2003; He et al., 2006; Li et al., 2005; de Torres et al., 2006; Zhang et al., 2007). The *Pseudomonas syringae pv tomato* (*Pst*) effectors avrRpm1 and HopM1 suppress callose deposition (DebRoy et al., 2004; Kim et al., 2005). Virulence effectors can have many different effects on the plant and many contribute to the suppression of resistance. For example, HopM1 suppresses callose deposition by inducing the degradation of the guanine exchange factor AtM1N7 which is required for vesicle trafficking to the cell surface (Nomura et al., 2006). HopM1 is also able to suppress the up-regulation of PR-1 (Ham et al., 2007).

R-gene mediated resistance

Plants have countered the suppression of basal resistance by evolving additional resistance responses, such as R gene-mediated resistance, (Maor and Shirasu, 2005) which is a robust response that relies on many different Resistance (R) genes to provide resistance to a range of taxonomically unrelated pathogens (Zhou et al., 1998). R genes encode receptors that detect specific pathogen–derived virulence factors such as *Pst* effectors or their effects on host

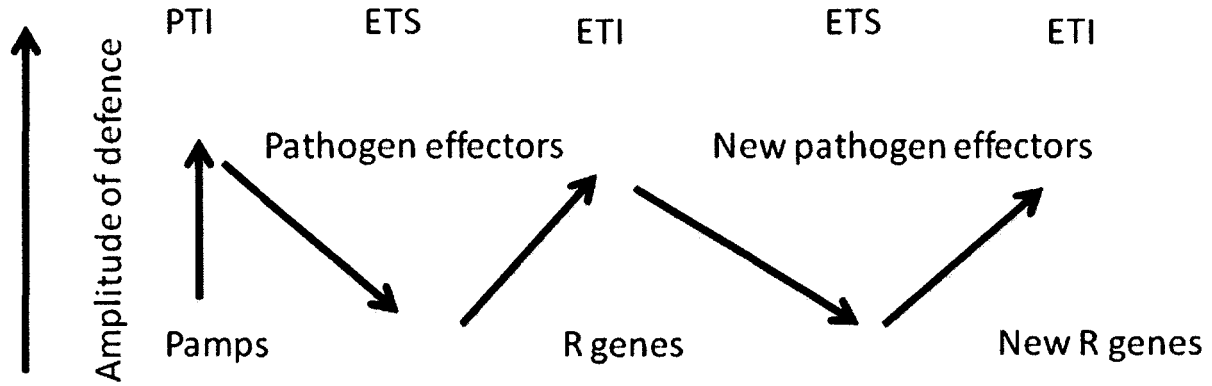
targets and therefore the resistance response is sometimes referred to as effector triggered immunity (ETI). When the pathogen contains a gene that encodes any determinant which can be recognized by the plant and induces ETI, the pathogen is avirulent on that host. Once detection has occurred, a defense response is mobilized which usually includes the SA-dependent induction of defense genes (Delaney et al., 1994) which leads to the production of *PR* proteins and phytoalexins (anti-microbial agents), cell wall modifications, and the hypersensitive response (HR) (Heath, 2000). The hypersensitive response is a form of programmed cell death, which is involved in restricting biotrophic pathogen growth and is usually accompanied by the generation of active oxygen species, phenolics, and the deposition of lignin-related materials, which reinforce the cell wall (Lamb and Dixon, 1997; Boldwell, 1999; Grant and Locke, 2000; and Rusterucci et al., 2001; Lee et al., 1995). Common to both basal resistance and R gene-mediated resistance as well as systemic acquired resistance (SAR), is the important regulator *EDS1* (enhanced disease susceptibility). The *EDS1* gene contains a catalytic site with homology to eukaryotic lipases (Falk et al., 1999). *EDS1* is essential for a proper manifestation of both basal resistance (Glazebrook, 1999; Feys and Parker, 2000) and SAR (Wiemer et al., 2005). *EDS1* is also required for R gene-mediated resistance if the activated R protein, such as RPS4, contains an N-terminal Toll/Interleukin receptor (TIR) domain with similarity to the intracellular domains of human and *Drosophila* toll receptor (Aarts et al., 1998; Parker et al., 1996; Parker et al., 1997; Botella et al., 1998;

Gassmann et al., 1999). When activated in response to detection of a pathogen, *EDS1* is found in the cytoplasm as a dimer or in complex with *PAD4* (phytoalexin deficient 4) and in the nucleus in complex with *PAD4* or *SAG101* (senescence associated gene 101). *EDS1* mediates downstream signaling of defense responses (Feys et al., 2001) including HR, oxidative burst, and accumulation of the signaling molecules nitric oxide and SA (McDowell et al., 2000). Expression analysis of the mutants *eds1* and *pad4* revealed that *EDS1* and *PAD4* are important for negative regulation of genes that contribute to the Jasmonic acid (JA) pathway (Broderson et al., 2006). PAD4 also contains a lipase domain and is required for SA and camelexin accumulation and *PR-1* expression (Jirage et al., 1999). A second important regulator for both SAR (Shapiro and Zhang, 2001) and R gene-mediated resistance (Century et al.,1995) is NDR1 (nonrace-specific disease resistance 1). *NDR1* is required for some R gene-mediated resistance responses where activated R genes such as RPS2 contain a coiled-coil domain or a leucine zipper in the N terminus of the protein (Century et al.,1995). *NDR1* encodes a small highly basic plasma membrane localized protein that functions downstream of ROS production but upstream of SA accumulation in the R gene-mediated resistance pathway (Coppinger et al.,2004).

The zig zag model is used to describe the interaction of PTI and ETI with virulent pathogens (Jones and Dangl, 2006) (Figure 1). Virulent pathogens such as *Pst* have "zigged" by evolving mechanisms such as virulence effectors to

suppress PTI. This phenomenom is referred to as effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). This causes the plant to "zag" by evolving ETI. As the pathogen and plant evolve together they will continue to zig and zag as the pathogen becomes able to elude the plant's defenses by evolving new virulence effectors and losing the effectors that were being detected by ETI and the plant responds with new mechanisms for resistance such as evolving new R genes that detect the new virulence effectors. Whether or not a plant is susceptible or resistant is calculated by the equation PTI + ETS – ETI (Jones and Dangl, 2006).

Figure 1: The zig zag model (modified from Jones and Dangl, 2006) illustrates the interaction of the plant immune system with virulent pathogens. PAMP triggered immunity (PTI) is the plant's initial line of defense against pathogens and is induced by PAMPs or MAMPs. Effecter triggered susceptibility (ETS) occurs when pathogens evolve mechanisms such as virulence effectors to overcome the plant's resistance response. Effecter triggered immunity (ETI) occurs when the plant evolves a mechanism to inhibit virulence effectors such as an R gene.



Systemic acquired resistance

Arabidopsis thaliana also possesses other induced forms of resistance. SAR takes place when a normally avirulent or certain necrotizing pathogens attempt to colonize the plant resulting in cell death and the production of the SAR mobile signal. The signal moves to distant tissues where it is perceived, and the plant is primed. *DIR1* (defective in induced resistance) is a putative lipid transfer protein that has been suggested to interact with a lipid-derived molecule to promote long distance signaling to systemic tissue (Maldonado et al., 2002). Upon subsequent infection with a virulent pathogen, defense responses are mobilized with greater speed. Resistance in the distant tissue is conferred through SA-mediated *NPR1* (non-expresser of *PR1*) and up-regulation of *PR* genes (Despres et al., 2000; Fan et al., 2002; Johnson et al., 2003; Kinkema et al., 2000; Subramaniam et al., 2001; Zhang et al., 2003). SAR results in a long-lasting enhanced defense response, which gives the host plant the ability to rapidly induce the defense response after secondary infection (Hammerschmidt, 1993; Kuc, 1983, Sequeira 1983)

Induced systemic resistance

Induced systemic resistance (ISR) is another well-studied inducible defense response. ISR is induced when soil-inhabiting nonpathogenic rhizobacteria colonize plant roots. Ethylene and JA (an oxylipin-like hormone derived from oxygenated linolenic acid,) are transiently increased (Ellis et al., 2002). JA is a signaling molecule involved in plant growth, the wound response,

and defense against necrotrophic pathogens, playing a role in multiple signaling pathways. When subsequent infection takes place, ISR occurs through an *NPR1*-dependent pathway and defense genes *PDF1.2* and *VSP2* are up-regulated. This type of resistance is effective against normally virulent pathogens such as some necrotrophic pathogens including the fungus *Alternaria brassicicola* as well as chewing insects (Ellis et al., 2002; Thomma et al., 1998). ISR differs from SAR in that SA and PR gene expression are unnecessary but is similar as resistance is conferred systemically and NPR1 is required (Ellis et al., 2002).

JA Pathway

Three well-characterized genes that are involved in the JA pathway have been isolated. *JAR1* (jasmonate-resistant 1) is thought to belong to the acyl adenylate-forming firefly luciferase super family which is composed of enzymes that activate the carboxyl group of a substrate for subsequent biochemical modification (Staswick et al., 2002). The JAR1 enzyme forms JA-amino acid conjugates using several amino acids including isoleucine (Ile) (Staswick and Tiryaki, 2004). JA can be conjugated with many different amino acids to form various jasmonates, which in turn control distinct processes (Laurie-Berry et al., 2006). Conjugation of JA by JAR1 is required for resistance to necrotrophic fungal pathogens such as *Pythium irregulare, Alternaria brassicicola, Botrytis cinerea* and the bacteria *Erwinia carotovara* (Staswick et al., 1998; van Loon et al., 1998; Clarke et al., 2000; Thomma et al., 1998; Stintzi et al., 2001; Vijayan et

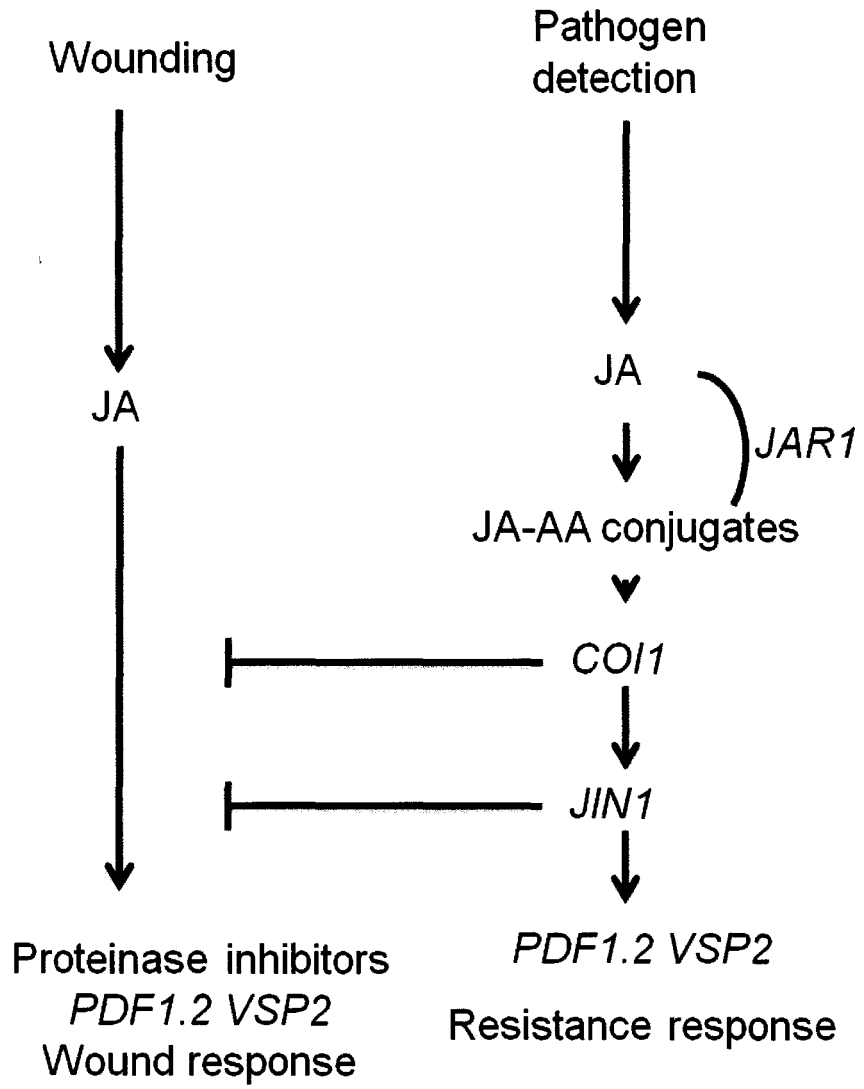
al., 1998; Norman-Setterbald et al., 2000). Another gene, *COI1* (coronatine insensitive 1) encodes an F-box protein, which is a component of an ubiquitin-like E3 complex that determines the substrate specificity of the complex (Xie et al., 1998; Devoto et al., 2005; Devoto and Turner, 2005; Yan et al., 2009). *COI1* is indispensable for JA signal transduction and is thought to be required for all aspects of JA signaling including pollen development (Feys et al., 1994) and defense against insects and necrotrophic pathogens (Penninckx et al., 1996; Thomma et al., 1998). *COI1* has homology to *Arabidopsis* TIR1, human Skp2, and yeast Grr1 (Xie et al., 1998). COI1 uses ubiquitination to target repressors (JAZ proteins) of JA-induced transcriptional responses for degradation (Xie et al., 1998; Staswick, 2008). The last gene, *JIN1* (jasmonate insensitive 1) encodes a MYC family transcription factor involved in mediating a subset of jasomonate-induced responses including disease resistance and the wound response and functions downstream of *JAR1* and *COI1* (Figure 2) (Boter et al., 2004; Lorenzo et al., 2004; Laurie-Berry et al., 2006). *JIN1* is repressed by the JAZ proteins and activated once the JAZ proteins are targeted for COI1 mediated degradation (Staswick, 2008).

The wound response

Wounding in *Arabidopsis* can take place through mechanical wounding such as through wind or rain damage or by herbivory through insects such as tobacco hornworm (Howe et al., 1996) and fungus gnats (McConn et al., 1997). In response to wounding the jasmonic acid pathway is induced (Figure 2),

including *JIN1,* and is sufficient to protect the plant from pathogens taking advantage of the wound site to gain entry into the plant (McConn et al., 1997). Defense mechanisms include the up-regulation of proteinase inhibitors that disrupt digestion in the insect's gut (O'Donnell et al., 1996).

Figure 2: JA is required for the wound response pathway and induced systemic resistance (ISR). Both the wound response pathway and the ISR pathway are *JIN1* and *COI1* dependent. The wound response leads to up-regulation of proteinase inhibiters and ISR leads to the up-regulation of defense genes *PDF1.2* and *VSP2*.



Age-related resistance

Age-related resistance (ARR) has been observed in diverse species, including rice, pepper, cowpea, tobacco, and *Arabidopsis thaliana* (reviewed in Develey-Rivière and Galiana, 2008). As some plants mature they become increasingly resistant to normally virulent pathogens. For example, increasing leaf age has been correlated with decreasing susceptibility in rice to pathogens such as *Xanthomonas campestris pv oryzae* (Koch and Mew, 1991) and *Pyricularia oryzae* (Roumen et al., 1992). Mature pepper plants accumulate the phytoalexin capsidiol in response to *Phytophthora capsici* (Hwang, 1995). ARR was also seen in adult cowpea when challenged with rust (Heath, 1993) and has been correlated with the transition to flowering or senescence and with the accumulation of secondary metabolites or defense proteins (reviewed in Develey-Rivière and Galiana, 2008). ARR coincides with flowering in *Arabidopsis*, whether the transition was delayed or accelerated (Rusterucci et al., 2005). *Arabidopsis* grown under a 16 hour photoperiod (long day) developed both ARR and started flowering early while plants grown under a 9 hour photoperiod (short day) developed ARR later and had delayed flowering (Rusterucci et al., 2005). Increased resistance to viral and fungal pathogens was observed in older leaves of flowering tobacco that were accumulating PR proteins such as PR-1, PR-2, and PR-3 and had an increased expression of genes involved in cell wall modifications (Fraser, 1972; Takahashi, 1972; Reuveni, et al., 1986; Wyatt et al., 1991; Hugot et al., 2004). Originally it was suspected that PR proteins might be

required for ARR because PR genes are up-regulated late in plant development (Fraser, 1981; Lotan et al., 1989; Buchanan-Wollaston, 1994; Hanfrey, 1996; Butt et al., 1998; Quirino et al., 1999) when ARR occurs (Kus et al., 2002). Currently, PR-*1* gene expression is not thought to be important for ARR due to the fact that *npr1* mutant plants defective for *PR-1* up-regulation in response to pathogen inoculation are still capable of displaying ARR (Kus et al., 2002; Cameron and Zaton, 2004). *PR-1* expression has also been shown to be reduced during *Arabidopsis* ARR compared to young plants that were inoculated with *Pst* (Kus et al., 2002). SA however, has been shown to increase in the intercellular space during ARR in *Arabidopsis* (Cameron and Zaton, 2004).

These observations led to the idea that SA may not be playing its traditional role as a signaling molecule and may have a unique function during ARR in *Arabidopsis* (Kus et al., 2002; Cameron and Zaton, 2004). Whether ARR affords broad-spectrum resistance to different pathogens has not been addressed for most plants, however mature tobacco plants become more resistant to the oomycetes *Peronospora tabacina* (Wyatt et al., 1991) and *Phytophthora parasitica* (Hugot et al., 1999) and to Tobacco Mosaic Virus (Fraser et al., 1981,Yalpani et al., 1991, 1993). Moreover recent studies have demonstrated that ARR is induced in mature *Arabidopsis* in response to virulent *Pseudomonas syringe pathovar tomato (Pst)* and *pathovar maculicola* (Kus et al., 2002) as well as *Hyaloperonospora arabidopsidis* (*H. arabidopsidis*) (Rusterucci et al., 2005). During ARR, *Pst*-induced chlorotic disease symptoms are reduced

in *Arabidopsis* and an HR-like response is not observed (Kus et al., 2002). In addition, bacterial growth is reduced 10 to 100-fold (Kus et al., 2002). Additionally, ARR is observed throughout the entire plant, not just in the older leaves (Kus et al., 2002). Information to date suggests that ARR is distinct from other induced disease resistance pathways such as SAR and ISR because NPR1 is not required for ARR (Kus et al., 2002).

The role of salicylic acid

Salicylic acid (SA) accumulation is required during many *R* gene-mediated and basal resistance responses and during SAR (Uknes et al., 1993; Vernooij et al., 1994; Delaney et al., 1994; Delaney et al., 1995). There is mounting evidence that during basal resistance and SAR, SA acts as a signaling molecule by inducing the reduction of key cysteines, leading to the translocation of reduced NPR1 into the nucleus. The reduced NPR1 then interacts with reduced TGA transcription factors, which subsequently up-regulate defense genes like *PR-1* (reviewed in Fobert and Despres, 2005).

The first evidence that salicylic acid accumulation is necessary for ARR came from plant lines that are deficient in SA accumulation and were also incapable of displaying the ARR response. SA deficient mutants include the transgenic line *NahG*, and mutants *sid1* and *sid2* (Gaffney et al., 1993; Nawrath and Metraux, 1999). The *NahG* line cannot accumulate SA due to expression of the salicylate hydroxylase gene (Gaffney et al., 1993). Salicylate hydroxylase, encoded by the *NahG* gene of *Pseudomonas putida* is a flavoprotein that

catalyzes the decarboxylative hydroxylation of SA, converting it to inactive catechol (Yamamoto et al., 1965). Catechol was shown to be inactive in tobacco as it could not induce SAR or prevent SA-dependent SAR gene induction (Friedrich et al., 1995). The *sid1* and *sid2* (SA induction deficient 1 and 2) mutants are deficient in SA accumulation because they do not synthesize SA, but do not actively degrade SA like the *NahG* lines do (Nawrath and Metraux, 1999). These two mutant lines are unable to accumulate SA, due to different single gene recessive mutations (Nawrath and Metraux, 1999). Both *sid* mutants show a reduction in *PR-1* gene expression and are defective in R gene-mediated resistance as they are susceptible to normally avirulent pathogens (Nawrath and Metraux, 1999; Nawrath et al., 2002). *SID1* is also allelic to the *eds5* (enhanced disease susceptibility) mutation (Nawrath and Metraux, 1999). *SID1* is predicted to have nine to eleven membrane-spanning domains with a coiled coil domain at the N-terminus encoding a putative chloroplastic multi drug and toxin extrusion transporter (MATE) that may function to transport chloroplastic SA into the cytoplasm (Nawrath et al., 2002).

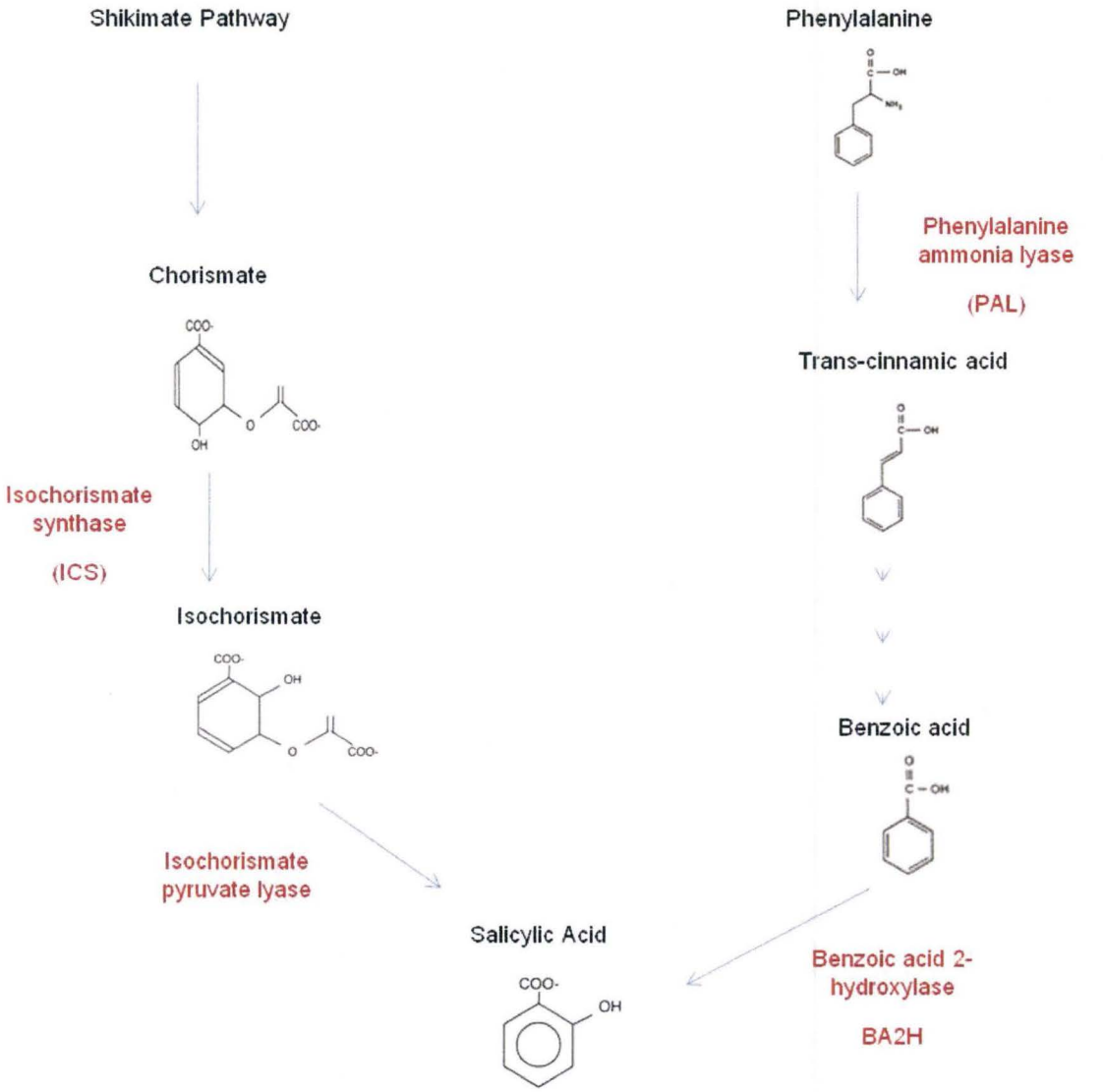
There are 56 other known MATE proteins in *Arabidopsis* some of which are involved in the transport of various organic molecules and are essential for such pathways as flavonoid biosynthesis (phenylpropanoid compounds and phytoalexins,) (Franke et al., 2002) and resistance to certain toxins (Debeaujon et al., 2001; Diener et al., 2001). Characterized members of the MATE family of proteins use a Na^+ efflux pump as the mechanism to transport organic molecules

(Morita et al., 2000). These observations led to an alternate hypothesis that *SID1* may also employ a Na^+ gradient to transport precursors for the biosynthesis of SA into the chloroplast where SA is synthesized (Nawrath et al., 2002). Expression of *SID1* is normally low, however it is strongly induced in plants exposed to stresses such as pathogen inoculation or exposure to ultra violet light (Nawrath et al., 2002). SA begins accumulating two to four hours after inoculations with *Pst* and levels are maintained for approximately two days (Nawrath et al., 2002). *SID1* transcript accumulation is normal in the *sid2* mutant (Nawrath et al., 2002).

SID2 encodes an isochorismate synthase gene, *ICS1*, which is expressed in tissues inoculated with pathogens as well as tissues displaying the SAR response (Nawrath and Metraux, 1999; Wildermuth et al., 2001). Isochorismate synthase is the penultimate enzyme in the synthesis of SA in the chloroplast (Metraux, 2002). SA is produced in higher plants through at least two pathways (Figure 1) including the shikimate-phenylpropanoid pathway where SA is synthesized from phenylalanine via cinnamic and benzoic acid and the isochorismate pathway (Sticher et al., 1997; Metraux, 2002). The phenotypes of *sid1* and *sid2* suggest, however, that pathogen-induced SA is synthesized through the isochorismate pathway (Figure 1) in the chloroplast, which is more typical of the prokaryotic pathway in bacteria (Metraux, 2002). Recent experiments have confirmed that ICS1 is the major contributor to pathogen-induced SA accumulation although a second isochorismate protein, ICS2, and

the phenylpropanoid pathway are capable of contributing small amounts (Garcion et al., 2008). The sequence of the *SID1* MATE protein is highly homologous to the DINF group of proteins found in an *E. coli* operon, providing further evidence of a prokaryotic origin of this section of the pathway (Kenyon and Walker, 1980).

Figure 3: Multiple SA biosynthesis pathways in *Arabidopsis* (Metroux 2002; Lee et al., 1995; Ribnicky et al., 1998).



During ARR, SA concentrations increase in the intercellular space from approximately 50 to 400 ng/g fresh weight or five- to ten-fold (Cameron and Zaton, 2004). This data correlates with anti-microbial activity in intercellular washing fluids as a 10-22% reduction in *in vitro Pst* growth (Cameron and Zaton, 2004). Removal of SA from the intercellular space using salicylate hydroxylase impairs ARR in wild type plants (Cameron and Zaton, 2004). Moreover, addition of SA to the intercellular space enhances the ARR response in wild type Columbia (Col-0) and rescues the ARR response in ARR deficient mutants (Cameron and Zaton, 2004). *NahG* plants were not rescued by infiltrating SA into the intercellular space likely due to the expression of the salicylate hydroxylase gene (Cameron and Zaton, 2004). No rescue or enhancement of ARR was observed when *sid* mutants or Col-0 were challenged 24 hours post SA infiltration as SA is absorbed into the cell over this time and is no longer present in the intercellular space (Kus et al., 2002; Cameron and Zaton, 2004). These results confirm the importance of intercellular SA accumulation during ARR and SA has been hypothesized to be transported to the intercellular space to act as an anti-microbial agent (Kus et al., 2002; Cameron and Zaton, 2004).

Elucidating the ARR pathway

Classical mutant screening was used to identify genes involved in ARR (Carviel et al., 2009). By disrupting the function of an ARR gene in a plant, that plant becomes defective for the ARR response. This ARR-defective phenotype

then identifies the gene and makes it possible to identify the mutant and wild type genotypes. Mutant screening was performed by inoculating mature (6 weeks post germination, wpg) neutron-mutagenized plants with 10^6 cfu/ml virulent *Pst* (Carviel et al., 2009). Plants displaying disease symptoms similar to ARR-defective *NahG* as compared to wild type Col-0 ARR-competent plants, were selected as potential mutants (M2 generation) and were re-screened in the M3 and M4 generations (Carviel et al., 2009). One mutant, *iap1-1* (important for the arr pathway), supported high levels of bacterial growth (~10^7 cfu/leaf disc (ld)) and was therefore compromised in ARR. *iap1-1* was genetically characterized by backcrossing with Col-0 to produce the F1 and F2 generations. Mature F1 plants supported intermediate levels of bacterial growth and symptoms (~$4x10^6$ cfu per leaf disc, ld) compared to wild type ARR-competent plants (~$6x10^5$ cfu/ld). F2 progeny segregated in a 1:2:1 ratio (chi square $p > 0.5$), ARR-competent (<$1.6x10^5$ cfu/ld) to intermediate ARR ($2x10^5$ to $8x10^5$ cfu/ld) to ARR-defective ($1x10^6$ to $3x10^7$ cfu/ld) respectively, suggesting that *iap1-1* is a semi-dominant mutation. A homozygous *iap1-1* plant line was isolated in the F3 generation and was used in all subsequent experiments.

Reverse genetics was also used to discover genes associated with ARR. An Affymetrix *Arabidopsis* microarray analysis was completed on plants displaying ARR. Affymetrix gene chips contain 25,000 *Arabidopsis* genes. The microarray analysis revealed that approximately one hundred genes were up-regulated and a similar number down-regulated in mature plants displaying ARR

compared to mock-inoculated controls (Carviel et al., 2009). T-DNA insertions in seven highly up-regulated genes were obtained from the Salk T-DNA collection. This collection contains T-DNAs randomly inserted into many *Arabidopsis* plants and these lines were sequenced by the Salk Institute to determine which genes contained a T-DNA insertion (potential knock-out line). A preliminary ARR assay showed that T-DNA mutants of seven putative ARR-associated genes displayed a reduced ARR response to *Pst,* six of which also displayed a reduced ARR response to *Hyaloperonospora arabidopsidis*. The four most promising genes were studied further and were confirmed to have a reduced ARR response (Carviel et al., 2009). These genes included a cytidine deaminase, (*CDA1*) uridine diphosphate-glusosyltransferase, *UGT85A1,* and two transcription factors *ANAC055* and *ANAC092.*

Research Objectives

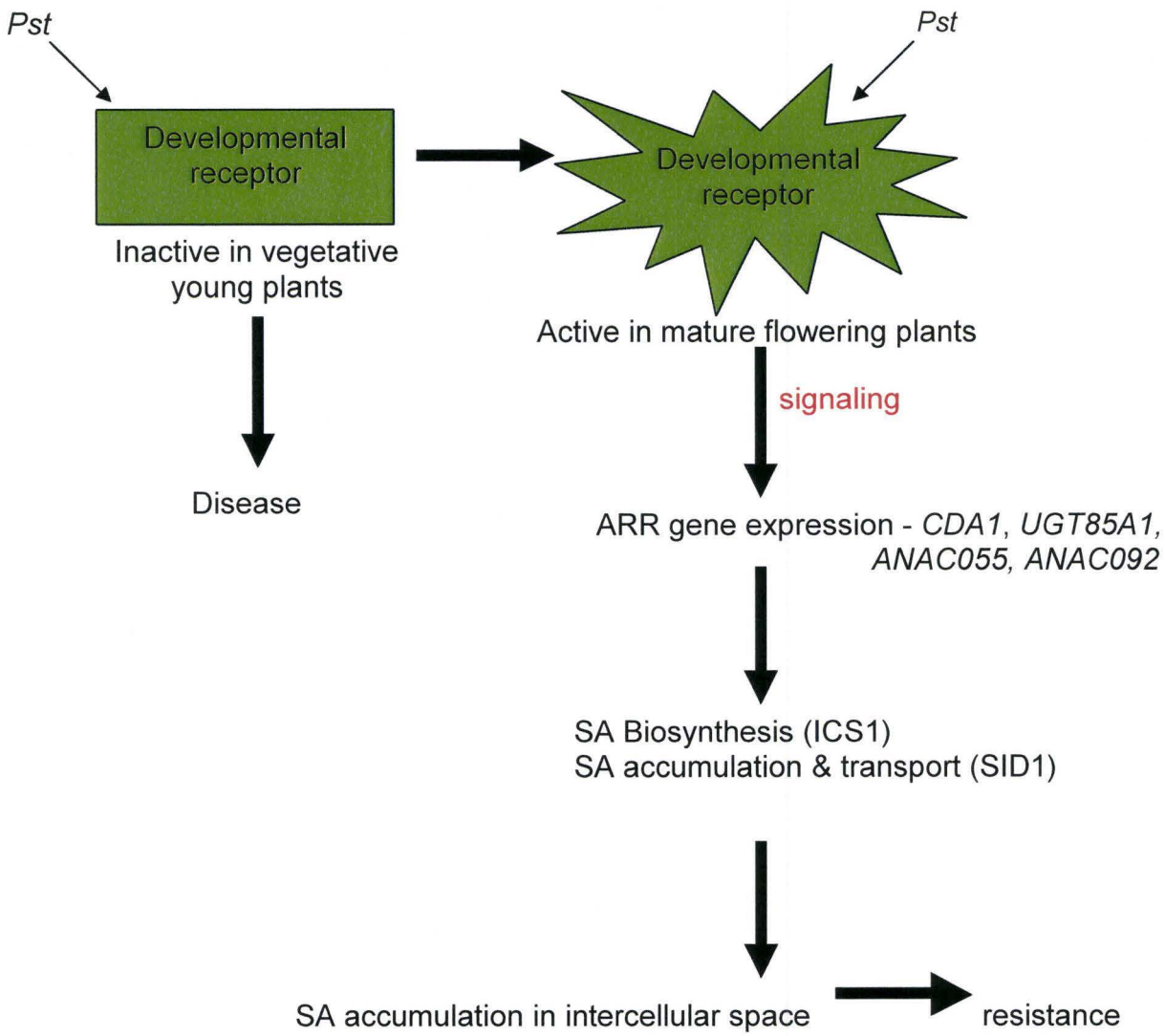
- Identifying genes important for ARR
- Characterization of *IAP1*
- Comparison of the ARR pathway with other disease resistance pathways

The overall goal of this thesis was to contribute to the elucidation of the ARR pathway. ARR is a particularly desirable disease resistance pathway to study because it may be a broad spectrum form of resistance (it has been shown to be effective against several diverse types of biotrophic pathogens) and is very effective as bacterial density is decreased by 100 fold in many cases compared to 5 – 10 fold observed in other broad spectrum disease resistance pathways

such as SAR. Knowledge of the ARR pathway could be used to increase disease resistance in crop plants. Unfortunately little is currently known about this pathway.

Below, (Figure 4,) is an ARR model based on previous SA rescue experiments, the ARR microarray, and subsequent results from ARR assays involving genes shown to be expressed during ARR according to the ARR microarray. In this model it was hypothesized that there is a developmentally regulated receptor that is activated as the plant matures and makes the transition from vegetative to flowering. Once the receptor is activated we hypothesize that it is required to perceive the presence of the pathogen, *Pst,* or is required in early signaling steps. The following cascade leads to the expression of ARR-associated genes including some of those identified in the microarray, leading to the accumulation of SA in the intercellular space and resistance to *Pst.*

Figure 4. ARR model based on the previous SA rescue experiments and the ARR microarray.



Chapter 2: Methods

Plant Growth Conditions

The *Arabidopsis thaliana* mutant *iap1-1* (*IAP1* registered on TAIR Gene symbol/name web page) was used in conjunction with controls, wild type Columbia (Col-0), transgenic *NahG* (K. Lawton, Syngenta, Research Triangle Park, NC) and *sid2* (C. Nawrath, University of Fribourg, Switzerland). Additional mutants that were examined include *eds1-1* (TAIR), *jar1-1,* and *jin1-1* (B. Kunkel, Washington University), *rlk* and cytochrome P450 T-DNA insertion lines (*Arabidopsis* Biological Resource Center, Ohio State University, USA, Alonso et al., 2003). Seeds were surface sterilized in 70% ethanol for two minutes, followed by sterilization in a solution containing 1.6% bleach, and 0.1% tween 20 for ten minutes, washed with sterile water five times, then vernalized at 4°C for at least two days before they were left to germinate on Murashige and Skoog (1962) medium for five to seven days. During this time, continuous light was provided. Seedlings at the two cotyledon stage were then transferred to soil, Sunshine Mix No. 1 (JVK) and moistened with 1 g/L 20-20-20 fertilizer. Temperature was maintained between 22°C and 24°C during a nine-hour photoperiod with an average light intensity of 150 $\mu Em^{-2} \cdot sec^{-1}$ as described previously (Rusterucci et al., 2005). Humidity ranged between 75% (winter) and 85% (summer).

Pst Disease Resistance Assays

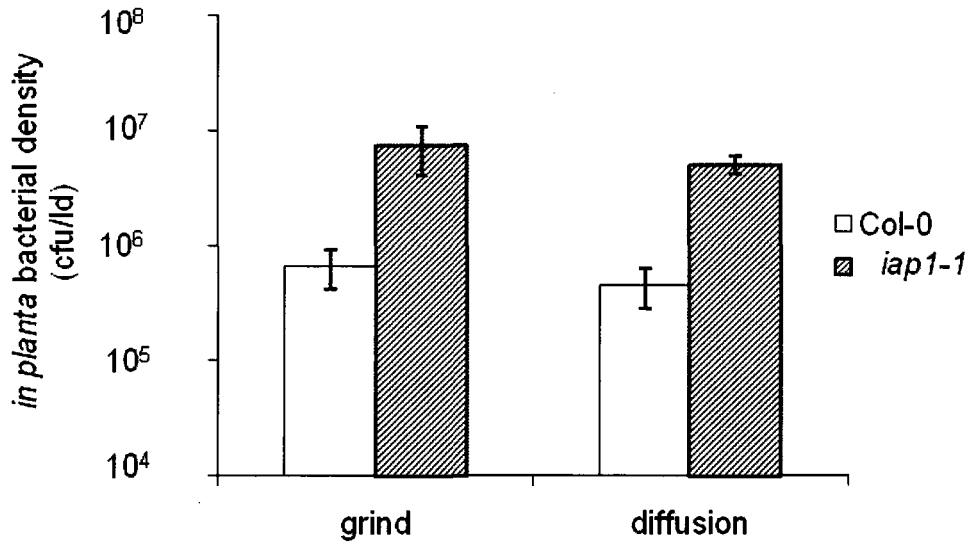
Arabidopsis plants were inoculated with virulent *Pseudomonas syringae pathovar tomato* (*Pst)* strain DC3000 (rifampicin and kanamycin resistant) or

avirulent *Pst(AvrRpt2)* (rifampicin and kanamycin resistant) obtained from Dr. Andrew Bent (University of Wisconsin at Madison) (Whalen et al., 1991) or *Pst(AvrRps4)* (rifampicin and kanamycin resistant) obtained from Dr. Gopal Subramaniam (Agriculture Canada). *Pst* was grown to mid-log phase in King's B media and kanamycin (50 μg/ml) over-night shaken at room temperature (22-24 °C), then centrifuged for seven minutes at 1000xg until bacteria were pelleted and media could be poured off. The bacteria were then re-suspended in 10 mM $MgCl_2$ to a final concentration of 10^6 or 10^7 colony forming units, (cfu/ml). Young (three weeks post germination, wpg) and mature (five or six weeks post germination, wpg) leaves on plants without visible inflorescence stems were inoculated and *in planta* bacterial levels were determined as previously described (Wolfe et al., 2000; Kus et al., 2002).

The previously used method for determining *in planta* bacterial levels which consisted of grinding inoculated leaves with a plastic drill bit was found to be time consuming and labor intensive. A more efficient method for determining *in planta* bacterial levels from Tornero and Dangl, 2001 was optimized for use in the Cameron lab and was shown to produce similar results to the grinding method (Figure 5). Eight leaf discs (4 mm diameter) were obtained as previously described (Wolfe et al., 2000; Kus et al., 2002) and were placed in 1.5 ml microfuge tubes with 1ml 0.2% silwet L-77 detergent in 10 mM $MgCl_2$. Microfuge tubes were collected in a 2 l erlenmeyer flask and shaken for 1h at 200 rpm at room temperature (22-24 °C). The detergent allowed bacteria to diffuse out of

the leaf discs replacing the previous grinding step. Photos of leaves were taken with a Hewlett Packard 5.6 MP 56X zoom digital camera.

Figure 5: The diffusion method of measuring bacterial density versus traditional grinding methods. Mature (6wpg) Col-0 and *iap1-1* were inoculated with virulent *Pst* (10^6 cfu/ml) and bacterial growth was measured 3dpi. Bacterial growth was compared between leaf discs that had been incubated with 0.2% silwet L-77 in 10mM $MgCl_2$ for one hour and control leaf discs which had been ground with a plastic drill bit. Bacterial growth is presented as the mean of three samples±SD.



Positional mapping of iap1-1

An F2 mapping population was created by crossing *iap1-1* (Col-0 background) with the *Landsberg erecta* (*Ler*) ecotype. The cross was confirmed in the F1 generation using indel markers (Jander et al., 2002). Indel markers use insertion or deletion differences between Col-0 and *Ler* as a way to differentiate between the two ecotypes. When DNA is PCR amplified at the site of an ecotype specific insertion or deletion the size of the product as observed on an agarose gel will reveal the DNA source. Screening for the *iap1-1* mutation was done by inoculating mature F2 plants with *Pst* and determining *in planta* bacterial levels after three days (see above). Plants were classified ARR-incompetent if bacterial growth was $\geq 10^7$ cfu/ld and ARR-competent if bacterial growth was $\leq 10^5$ cfu/ld. Heterozygotes were discarded.

Indel markers (Jander et al., 2002) were used to positionally map the *iap1-1* mutation (Konienczny and Ausubel, 1993). Indel markers at sites of insertions or deletions in the Col-0/*Ler* genomes were PCR amplified and products were visualized on 2% agarose gels (Jander et al., 2002). *IAP1* is believed to be located on the long arm of chromosome four where more ARR wild type plants contain *Ler* DNA and more ARR-defective plants contain *iap1-1* DNA than is statistically probable to occur by chance.

ARR screen using fluorescent Pst

Hundreds of plants were screened for ARR during the mapping of *IAP1*. As was previously mentioned the ARR assay is time consuming and labor

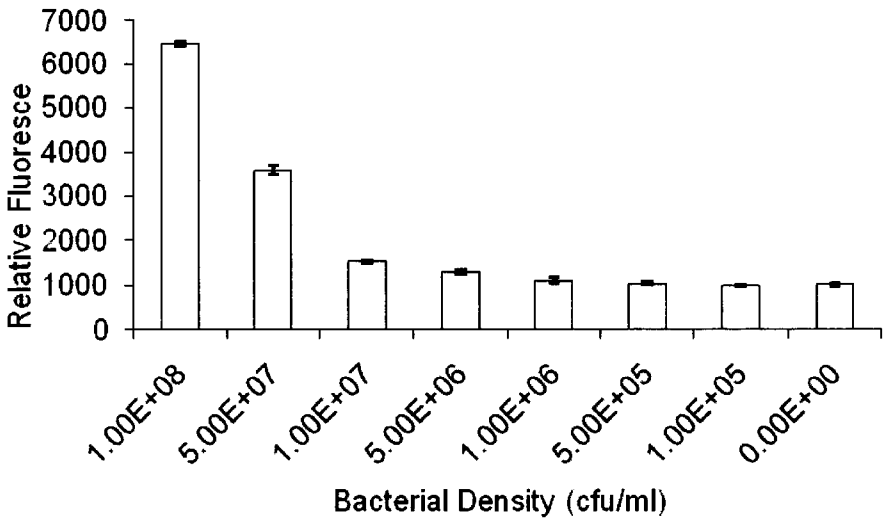
intensive. A new ARR screen was developed during the characterization of the F2 mapping population to reduce the amount of time and labor required to test plants for ARR. A virulent strain of *Pst* was transformed with the plasmid pKT-trp obtained from (Dr. S. Lindow, Berkeley), which contains kanamycin resistance and the reporter gene *GFP* constitutively driven by the *trp* promoter of *Salmonella typhimurium* (Miller et al., 2000; Monier and Lindow, 2003). *Pst* was grown to mid-log phase in King's B liquid media and kanamycin (50 µg/ml,) over-night shaken at room temperature (22-24 °C), and then diluted to 10^6 colony-forming units, (cfu/ml) in 10 mM $MgCl_2$. A sterile 1ml syringe, (without a needle,) was used to inoculate the abaxial side of the leaf in mature (6wpg) F2 plants to be screened along with both wild type (Col-0) and mutant (*iap1-1*) controls. After three days leaf disks (4mm diameter, eight per replicate) were collected from inoculated leaves and transferred to a microfuge tube containing 1 ml 0.2% silwet L-77 in 10mM $MgCl_2$. Microfuge tubes were collected in a two-litre flask and shaken on a rotary shaker for one hour at 200 rpm (Tornero and Dangl, 2001). After one hour, 100 µl of liquid solution was transferred to an opaque 96 well plate and a fluorometer was used to measure fluorescence (485nm excitation filter/535nm emission filter for one second). Mutant F2 plants were identified as the plants with the highest levels of fluorescence and similar fluorescence levels as mutant controls.

In this way serial diluting and plating bacteria from leaf discs is replaced with measuring fluorescence of leaf discs. A fluorometer can accurately measure

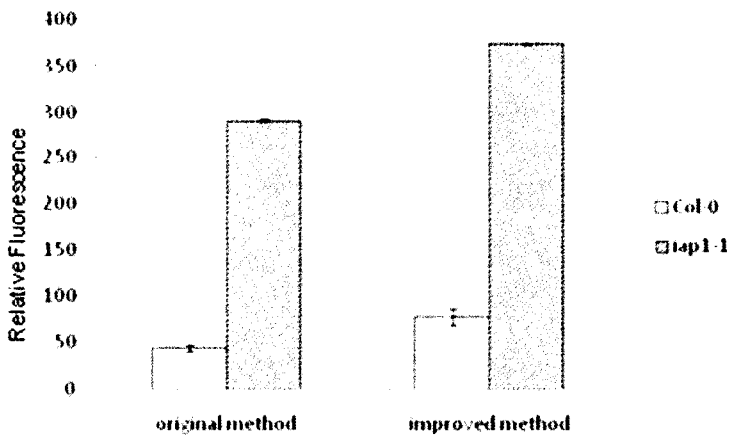
fluorescence in 96 samples simultaneously, at the same time eliminating the two day period it takes for bacteria to grow on plates. This method works well when the bacterial density is greater than 10^7 cfu/ml (Figure 6a) which allows for the identification of *iap1-1* homozygous mutants. In order to differentiate between wild type and heterozygotes *Pst* containing pDSK-GFPuv (Wang et al., 2007) was substituted in the hopes that it contained stronger *GFP* expression than *Pst* pKT-trp. Unfortunately results obtained using *Pst* containing pDSK-GFPuv were similar to results obtained using *Pst* pKT-trp. To increase GFP levels enough to detect the differences between wild type and heterozygotes the amount of leaf discs used was increased from 8 to 16 and the amount of 10mM $MgCl_2$ was decreased from 1ml to 800µl (Figure 6b).

Figure 6: Use of fluorescent *Pst* to quantify bacterial growth. Bacterial density was estimated by the level of fluorescence resulting from *Pst* expressing *GFP.* A) The original method used eight leaf discs and 1ml of 10mM $MgCl_2$ buffer. B) The improved method used 16 leaf discs and 800µl of 10mM $MgCl_2$ buffer. Bacterial density is presented as the mean of three samples±SD.

A)



B)



SA Infiltration Experiments, Intercellular Washing Fluid (IWFs) Collection, and SA determination

SA infiltration of *Arabidopsis* leaves has previously been shown to rescue the ARR defect in some ARR mutants (Cameron and Zaton, 2005). SA infiltration was performed on the *iap1-1* and *eds1-1* mutants in an attempt to rescue ARR function. The *iap1-1* and *eds1-1* plants were infiltrated with 0.1mM SA dissolved in water with a needless syringe on the abaxial side of the leaf at five weeks post germination (wpg). Five hours after infiltration, these same leaves were challenged with virulent *Pst* (10^6 cfu/ml). *In planta* bacterial levels were monitored three days after challenge and compared with controls, which included *iap1-1* or *eds1-1* infiltrated with sterile water, Col-0 infiltrated with 0.1mM SA, and Col-0 infiltrated with sterile water. A second experiment which included an additional control of both *iap1-1* and Col-0 infiltrated with 0.1mM SA 24 hours prior to *Pst* inoculation was compared with sterile water-infiltrated plants 24 hours prior to *Pst* inoculation.

IWFs from mature *iap1-1* plants were collected to determine if they possessed anti-microbial activity, as compared with wild type Col-0 plants. IWF collections were performed as described previously (Cameron and Zaton, 2004; Kus *et al.*, 2002). Col-0 and *iap1-1* plants were inoculated with 10^6 cfu/ml *Pst* in order to induce the ARR response. Col-0 and *iap1-1* controls were mock-inoculated with 10mM $MgCl_2$. After 24 hours, inoculated leaves were harvested and surface sterilized using a 50% ethanol and 0.05% bleach solution. Leaves

were then vacuum infiltrated with sterile water for 25 minutes. Finally, to obtain the intercellular washing fluids, leaves were blotted dry and centrifuged at 1000xg for 30 minutes in 50ml syringes fitted into microfuge tubes. The IWFs were filter sterilized to remove the previously inoculated *Pst*. These sterile IWFs were incubated with a known concentration of *Pst* for one hour and bacterial growth was monitored as described above, three days later and compared with controls that were incubated with sterile water. SA levels were measured using gas chromatography mass spectrometry (Schmelz et al., 2004) by Dr. Wolfgang Moeder, University of Toronto.

IWFs were also collected from young plants in order to measure SA accumulation during R gene-mediated resistance and in response to *Pst*. Col-0 and *iap1-1* plants (4 wpg) were inoculated with virulent *Pst* or avirulent *PstAvrRpt2* (10^6 cfu/ml) or were mock-inoculated with 10mM $MgCl_2$ or left untreated. Leaves were harvested at 12, 24, and 48 hpi and IWFs were collected as described above. The biosensor ADPWH_*lux* is a non-pathogenic soil bacterium that has been modified to emit a proportional amount of luciferase in the presence of SA (Huang et al., 2006) and was employed to measure free SA and SAG as described previously (DeFraia et al., 2008). This method was used as it requires no expensive equipment. ADPWH_*lux* was grown in Luria Broth (LB) overnight shaken at room temperature (22-24 °C). It was diluted to 0.4 OD_{600} with fresh LB to be incubated with IWFs or ground leaf tissue with IWFs

removed for one hour at 37°C in an opaque 96 well plate (DeFraia et al., 2008). Luminescence was measured and used to calculate the amount of SA.

Analysis of ARR-Related Gene Expression using RT-PCR

Leaf samples were collected from *iap1-1* and wild type Col-0 inoculated with virulent *Pst* (10^6 cfu/ml) as well as untreated controls to analyze gene expression. Samples were collected at various time points between 7 and 24 hours post inoculation from mature (6 wpg) plants.

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). cDNA was synthesized using the SuperScript III (Invitrogen) reverse transcriptase kit, according to the manufacturer's instructions. Twenty-eight cycles of PCR were used with gene specific primers listed in table 1 and actin was used as the constitutive internal control.

Table 1: Primers used in reverse transcriptase polymerase chain reaction experiment.

Gene	Primer Sequence
CDA1 At2g19570	TCCAAAGAAGCAGAATCCGC
	GTCTCTAATAACAACCTCGCC
UGT85A1 At1g22400	GTTTGTGTTCCATATCCGGC
	GAACCATAGCCTCCTCTCC
ANAC055 At3g15500	ATGGGTCTCCAAGAGCTTGA
	TCAAATAAACCCGAACCCAC
ANAC092 At5g39610	ATGGATTACGAGGCATCAAG
	TCAGAAATTCCAAACGCAAT
RLK At2g02710	CCAGTTCGTAATGCTTCAGG
	GCATGTCACCATCAATGAGC
SID1 At4g39030	CCTTTCTTCATGGCGTTGTCTG
	TTCTCCACCGTGTATGGACTCG
ICS1 At1g74710	AACCAGTCCGAAAGACGACCTC
	CAAATTCACTCTCCTCGCCACC
EDS1 At3g48090	GCCAAACGGAATGATTGCAGA
	TCCATTCTCCAAGCATCCCTT

Measuring HR

Col-0 and *iap1-1* leaves inoculated with *Pst(AvrRpt2)* (10^7 cfu/ml) or mock inoculated with 10mM $MgCl_2$ as described above were harvested at 24hpi and immediately submersed in trypan blue staining solution (0.02g trypan blue, 8% phenol, 8% glycerol, 8% lactic acid, 8% water, 67% 95% ethanol). Leaves were boiled for one minute in the staining solution and left overnight followed by destaining in 70% ethanol to reveal cell death. Images were captured using the McMaster University biology departmental confocal microscope (Nikon eclipse TE2000-S) at 10x magnification and Nikon digital camera DXM1200F. Ion leakage was measured (using conductivity) to quantify HR. Forty-eight medium sized leaves were first rinsed to remove ion leakage resulting from damage at ends cut from the petiole and then were added to 80ml of distilled water. The

conductivity of the water was measured hourly using an YSI model 556 probe from 7 to 13hpi.

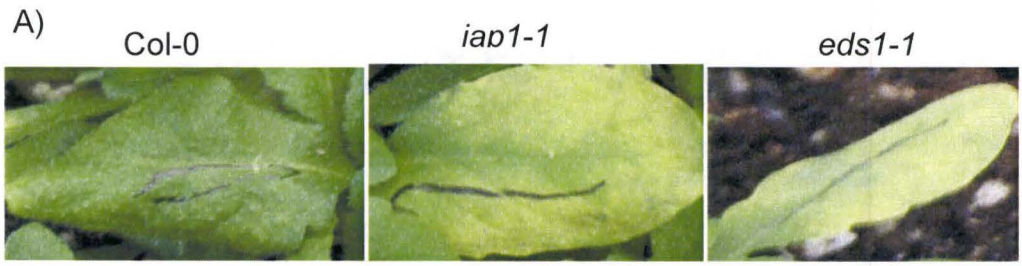
Chapter 3: Are other defense response genes required for ARR

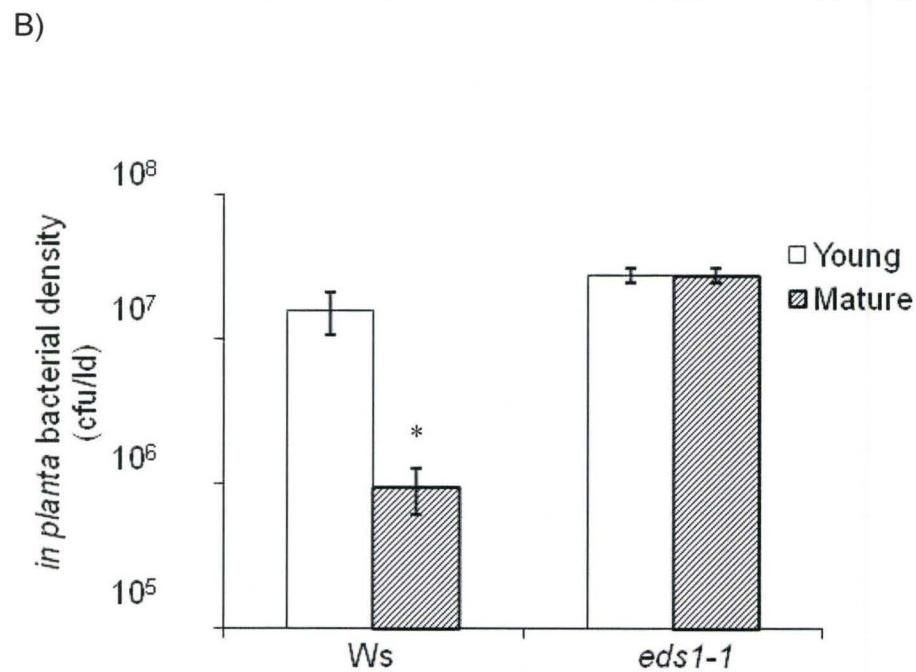
Genes involved in other disease resistance pathways and genes that were previously shown to be expressed during ARR were assayed for ARR. ARR assay results for *eds1-1, iap1-1, jar1*, and *jin1* were published in Carviel et al., 2009. All of the experiments presented in this chapter were completed by the author.

eds1-1 is ARR defective

EDS1 (enhanced disease susceptibility) is an essential regulator necessary for the accumulation of SA, which acts as a signaling molecule to up-regulate downstream defenses (Feys et al., 2001; Feys et al., 2005). Since EDS1 is involved in SA accumulation (Feys et al., 2001; Feys et al., 2005), it was hypothesized that EDS1 may play a role in ARR as intercellular SA accumulation is believed to be important for ARR (Cameron and Zaton, 2004). *eds1-1* and Ws wild type plants were assayed for ARR by inoculating young and mature plants with *Pst* (10^6 cfu/ld). This experiment allows for the comparison of *Pst* levels in young ARR-incompetent plants with mature plants displaying ARR. A significant decrease in bacterial growth (Student's t test $P<0.05$) was not observed in *eds1-1*, as bacterial density remained high (~10^7) in both young and mature plants (Figure 7b). Conversely bacterial density was reduced 17-fold in mature wild type plants displaying ARR compared to young plants. This suggests that *EDS1* is required for the ARR response.

Figure 7. *eds1-1* is deficient for ARR. A) Disease symptoms in *eds1-1* compared to controls Col-0 and *iap1-1*. Leaves were inoculated with 10^6 cfu/ml *Pseudomonas syringe* pv. *tomato* (*Pst*) and photographed at 3 days post-inoculation (dpi). B) *eds1-1* and Ws were inoculated with 10^6 cfu/ml *Pst* at 3 and 6 wpg. *In planta* bacterial levels (cfu/leaf disc (ld)) were monitored 3 dpi and are presented as the mean ± SD of three samples. Stars indicate a significant decrease in bacterial density (Student's t test $P<0.05$). This experiment was repeated twice with similar results.





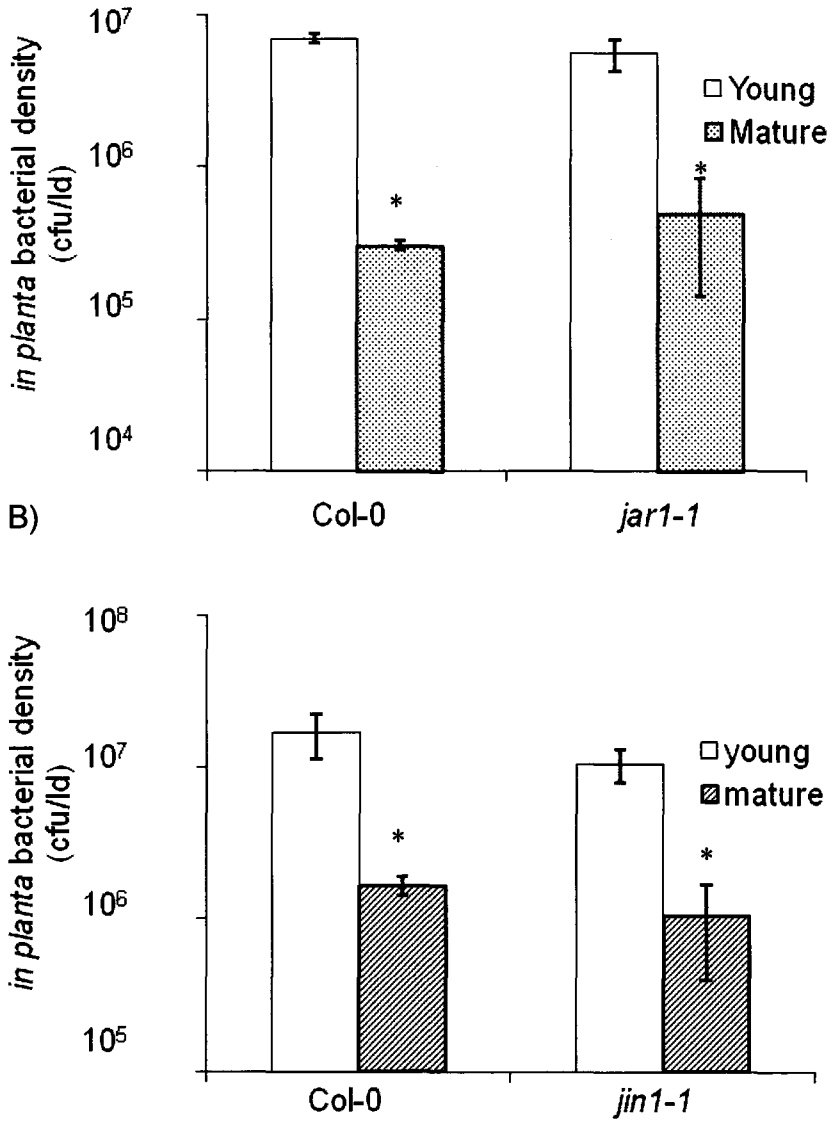
The ARR response occurs in jar1-1 and jin1-1

Jasmonic acid (JA) and related metabolites (such as JA-Ile) are lipid-derived compounds which act as signals in defense to some pathogens (often necrotrophic) and in the wound response to insects, as well as in plant growth and development (reviewed in Wasternack, 2007). Work to date suggests that SA acts as an anti-*Pst* agent in the intercellular space during ARR rather than as a signal for up-regulation of defense genes like *PR-1* (Kus et al., 2002; Cameron and Zaton, 2004). Therefore other signaling molecules such as JA may be required for ARR signaling. To test this hypothesis, two JA mutants, *jar1-1* and *jin1-1* were assayed for ARR competence.

JAR1 encodes a JA-amino acid synthetase that activates JA for optimal signaling in *Arabidopsis* by conjugating it to amino acids such as isoleucine (Staswick and Tiryaki, 2004). Therefore *jar1-1* mutants do not accumulate activated JA-Ile and are defective for downstream JA-Ile-induced defense signaling via COI1 (Staswick and Tiryaki, 2004). Col-0 and *jar1-1* mutants were assayed for ARR competence by inoculating both young and mature plants with *Pst* (Figure 8a). A ten-fold decrease in bacterial density was observed in mature compared to young Col-0 and *jar1-1* plants. This significant decrease in bacterial growth (Student's t test $P<0.05$) in mature plants suggests that *jar1-1* mutants maintain a functional ARR pathway despite the disruption in JA signaling.

Studies indicate that the phytotoxin coronatine produced by the *P. syringae* group of pathovars (including *Pst*) acts as a molecular mimic of JA-Ile (Krumm et al., 1995; Staswick and Tiryaki, 2004; Brooks et al., 2005) to elicit the JA signaling pathway downstream of JAR1 via COI1 and JIN1 which leads to suppression of SA-mediated defenses thereby favouring *Pst* growth (Laurie-Berry et al., 2006). Therefore, although studies with *jar1-1* and *Pst* will reveal if a JA-dependent response requires JA-Ile, it cannot establish if an intact JA signaling pathway is required (Laurie-Berry et al., 2006). Instead studies with jasmonate insensitive mutants like *jin1-1* are necessary. *JIN1* is a MYC2 transcription factor (Lorenzo et al., 2004) that functions downstream of JAR1-synthesized JA-Ile and *Pst*- produced coronatine and therefore is required for JA defense signaling even in the presence of coronatine-producing pathogens (Laurie-Berry et al., 2006). Young and mature Col-0 and *jin1-1* plants were tested for their ability to display ARR using the ARR assay (Figure 8b). A significant ten-fold decrease (Student's t test $P<0.05$) in bacterial growth in mature compared to young Col-0 and *jin1-1* plants confirms that *jin1-1* mutants have an intact ARR pathway suggesting that JIN1 and JA signaling are not required for ARR.

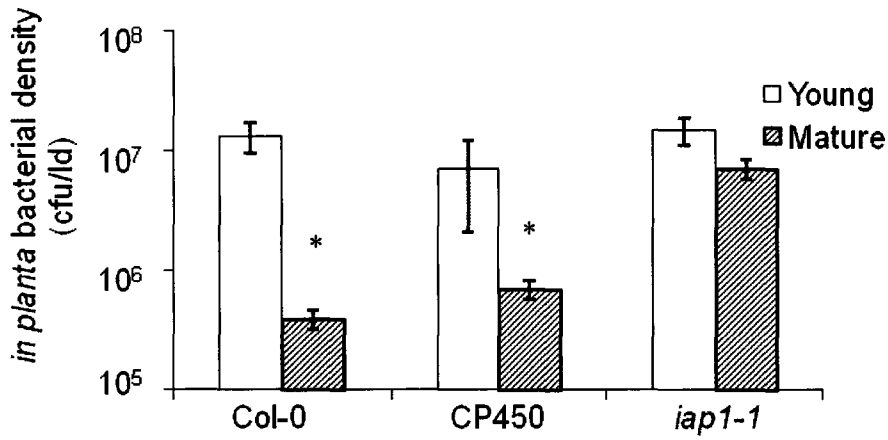
Figure 8. *jar1-1* and *jin1-1* display ARR. *jar1-1* (A), *jin1-1* (B), and Col-0 were inoculated with 10^6 cfu/ml *Pst* at 3 and 6 wpg. *In planta* bacterial levels were monitored 3 dpi and are presented as the mean ± SD of three samples. Stars indicate a significant decrease in bacterial density (Student's t test $P<0.05$). Results were repeated once for *jin1-1* and twice for *jar1-1* with similar results.



The ARR response occurs in Cytochrome P450 (CYP71A13) T-DNA insertion lines

Cytochrome P450 genes make up a large family of *Arabidopsis* genes involved in plant defense. A microarray experiment (Carviel et al., 2009) demonstrated that *CYP71A13* is up-regulated during ARR, suggesting that it is involved in the ARR pathway. Col-0, *iap1-1*, and plants with a heterozygous *CYP71A13* T-DNA insertion were assayed for ARR by inoculating young and mature plants with *Pst* (10^6 cfu/ml). A similar significant decrease in bacterial growth (Student's t test $P<0.05$) was observed in *CYP71A13* plants and Col-0 controls between young and mature plants (Figure 9). This suggests that two copies of the *CYP71A13* gene are not required for the ARR response.

Figure 9: *CYP71A13* T-DNA insertion plants display ARR. *CYP71A13* T-DNA plants, *iap1-1,* and Col-0 were inoculated with 10^6 cfu/ml *Pst* at 3 and 6 wpg. *In planta* bacterial levels were monitored 3 dpi and are presented as the mean ± SD of three samples. Stars indicate a significant decrease in bacterial density (Student's t test $P<0.05$).



The ARR response occurs in RLK T-DNA insertion lines

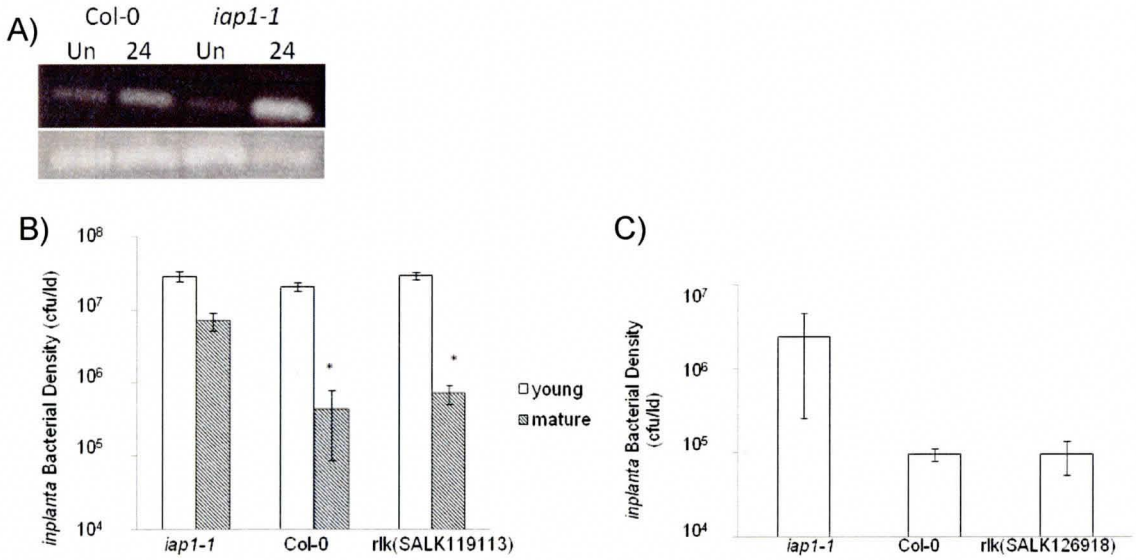
A putative receptor like kinase (RLK) (At2g02710) was shown to be up-regulated during ARR in an ARR microarray experiment (Carviel et al., 2009) and confirmed using RT-PCR (Figure 10a). This RLK is a putative blue light sensor, which contains a PAS motif (Ogura et al., 2007). Little is known about this *Arabidopsis* RLK, however a similar PAS motif receptor may play a role in development in mice (Lahvis and Bradfield, 1998; Walisser et al., 2004) and has further been implicated in the protection against physiological stress in mammals (Zhou et al., 1997). PAS domain proteins are cytoplasmic (Taylor, B.L., and Zhulin, 1999) and stimuli such as binding of a ligand or sensory signals (redox changes and light) activate the PAS containing proteins, which then translocate into the nucleus and influence transcription of target genes (reviewed in Hankinson 2005; Zhulin et al., 1997).

RLK (At2g02710) is located on the short arm of chromosome two in the same region that early mapping experiments suggested *IAP1* was located, thus it was originally speculated that this RLK might be *IAP1*. As similar PAS domain containing proteins are able to induce gene transcription RLK appears to have the potential to act as a positive regulator. RLK is also up-regulated during ARR and therefore was a candidate gene for *IAP1.*

RT-PCR revealed that RLK is expressed similarily in wild type and *iap1-1* mutants (Figure 10a) suggesting that the RLK promoter has not been affected by the *iap1-1* mutation. Sequencing revealed that *iap1-1* mutants also contain an

intact RLK sequence. Possessing both a functional RLK promoter and an intact coding sequence in *iap1-1* suggests that IAP1 is not RLK. However since RLK is expressed during ARR, an RLK T-DNA insertion line (SALK 119133) was tested for its ability to display ARR to determine if this gene should be examined further for its role in the ARR pathway. The SALK 119133 T-DNA insertion line is expected to contain a homozygous T-DNA insertion in the promoter. To ensure the T-DNA insertion was present, seeds were plated on media which contained kanamycin. The T-DNA insertion encodes kanamycin resistance therefore, it is expected that only those seedlings that contain the T-DNA insertion will survive. Moreover, many seedlings did not survive suggesting that only plants containg a T-DNA insertion survived to be used in the ARR assay. Col-0, *iap1-1*, and RLK T-DNA insertion plants were assayed for ARR by inoculating young (3wpg) and mature (6wpg) plants with *Pst* (10^6 cfu/ml). A 40-fold decrease in bacterial density was observed in mature *rlk at2g02710* versus young *rlk at2g02710*, suggesting that RLK is not required for ARR (Figure 10b). A second experiment was completed by inoculating mature (6wpg) plants with a different T-DNA in RLK (SALK 126918) with *Pst* (10^6 cfu/ml) (Figure 10c). SALK 126918 T-DNA insertion lines are expeccted to contain a T-DNA insertion in an exon and were also screened using kanamycin to obtain seedlings which contained the insertion. Bacterial density was not significantly different from wild type controls (anova, $P<0.05$) also suggesting that this RLK is not required for ARR.

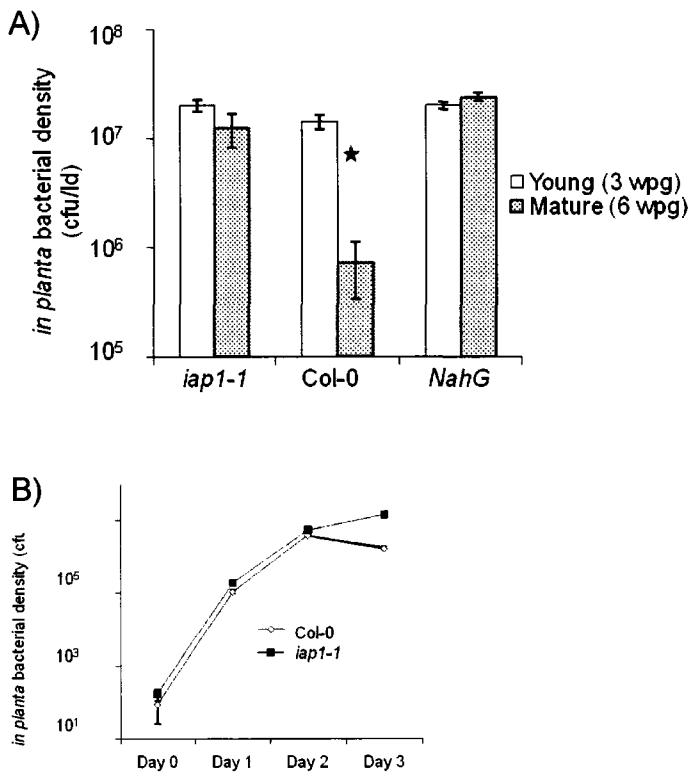
Figure 10. RLK is up-regulated during ARR and RLK T-DNA insertion lines (SALK 119133 and SALK 126918) display ARR. A) RT-PCR analysis was performed to monitor RLK expression in Col-0 and *iap1-1*. Mature (6 wpg) Col-0 and *iap1-1* leaves were collected 24 hpi with *Pst* (10^6 cfu/ml) along with untreated controls (Un). *ACTIN* was used as a loading control. 25 cycles of PCR was used. B) Col-0, *iap1-1,* and an RLK T-DNA insertion line (SALK 119133) were inoculated with 10^6 cfu/ml *Pst* at 3 and 6 wpg. *In planta* bacterial levels were monitored 3 dpi and are presented as the mean ± SD of three samples. Stars indicate a significant decrease in bacterial density (Student's t test $P<0.05$). C) Col-0, *iap1-1,* and an RLK T-DNA insertion line (SALK 126918) were inoculated with 10^6 cfu/ml *Pst* at 6 wpg. *In planta* bacterial levels were monitored 3 dpi and are presented as the mean ± SD of three samples.



IAP1-1 is required for ARR

Since high (~10^7 cfu/ml) *Pst* levels were observed in both young (3 wpg) and mature (6 wpg) plants, *iap1-1* is considered to be an ARR loss of function mutant (Figure 11a). In standard ARR assays, bacterial levels are measured at 3 dpi (days post inoculation), therefore bacterial growth was monitored over three days to determine when the ARR defect in *iap1-1* is first observed. Bacterial growth in Col-0 and *iap1-1* was similar at 0, 1, and 2 dpi, demonstrating that the number of bacteria infiltrated and *Pst* growth over 2 days is similar in both. At 3 dpi, Col-0 plants displayed ARR, whereas *Pst* levels were 10-fold higher in ARR-defective *iap1-1* (Figure 11b).

Figure 11. Disease resistance in *iap1-1*. A) Col-0, *iap1-1* and *NahG* were inoculated with 10^6 cfu/ml *Pst* at 3 and 6 wpg (weeks post germination). *In planta* bacterial levels were monitored 3 dpi (days post inoculation) and are presented as the mean ± SD of three samples. Stars denote a significant decrease in bacterial growth between young (3 wpg) and mature (6 wpg) plants (Student's t test $P<0.05$). This experiment was repeated three times with similar results. B) *In planta* bacterial growth in mature *iap1-1* and Col-0 plants (6 wpg) was measured over 3 dpi (10^6 cfu/ml *Pst)* and is presented as the mean of three samples ± SD. By day three, bacterial density in Col-0 is significantly lower (Student's t test $P<0.05$). This experiment was repeated with similar results.



Chapter 4: The role of *IAP1* during ARR, Basal Resistance, and R gene-mediated resistance

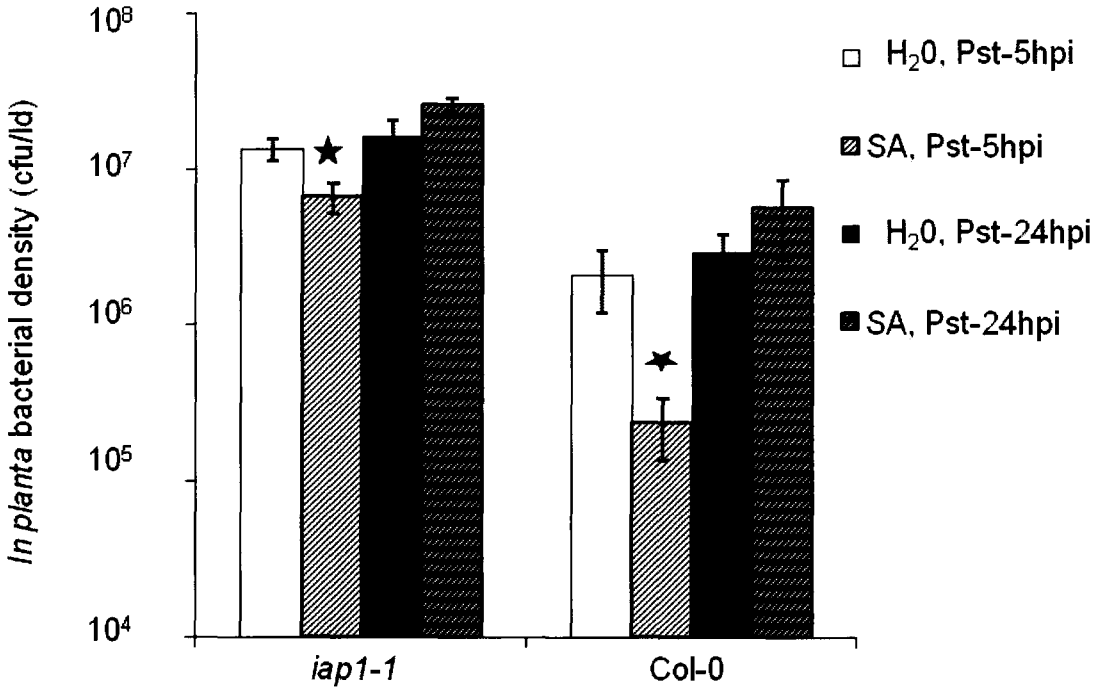
Basal resistance in *iap1-1*, ARR gene expression in *iap1-1*, and SA accumulation in mature *iap1-1* results were published in Carviel et al., 2009. R gene-mediated resistance and SA accumulation in young *iap1-1* results are currently being prepared for publication. GC/MS analysis was completed by Dr. Wolfgang Moeder, University of Toronto. All other experiments presented in this chapter were completed by the author.

SA infiltration partially rescues the iap1-1 ARR defect

Previous work suggests that the ARR pathway leads to an accumulation of SA in the intercellular space where it may act as an anti-microbial agent during ARR (Kus et al., 2002; Cameron and Zaton, 2004). If *IAP1* is upstream of SA accumulation in the ARR pathway and SA accumulation is a key component of ARR, then SA addition to the intercellular space prior to *Pst* inoculation should rescue the ARR defect in *iap1-1*. SA (0.1 mM) was infiltrated into mature *iap1-1* and Col-0 plants 5 and 24 hours prior to inoculation with *Pst* (10^6 cfu/ml). *iap1-1* and Col-0 controls were infiltrated with water and then inoculated with *Pst,* 5 and 24 hours later. ARR was enhanced 20-fold in Col-0 plants inoculated with *Pst* at 5 hours post SA infiltration (hpSAi) compared to 24 hpSAi or in Col-0 control plants infiltrated with water (Figure 12). *Pst* levels were reduced 2-fold in *iap1-1* plants challenged with *Pst* at 5 hpSAi compared to 24 hpSAi or in the water-infiltrated *iap1-1* control (Figure 12). In two additional experiments, *Pst* levels

were reduced 2- and 4-fold in *iap1-1* inoculated with *Pst* at 5 hpSAi compared to the water-infiltrated controls. Enhancement of ARR was not observed in Col-0 and *iap1-1* 24hpSAi as SA gets absorbed into the cell and enhancement of ARR was only observed when SA was still present in the intercellular space (5 hpSAi) (Cameron and Zaton, 2004). *Pst* levels were reduced in *iap1-1* to a lesser extent compared to Col-0, therefore the ARR defect in *iap1-1* was partially restored by intercellular SA application suggesting that IAP1 is upstream of intercellular SA accumulation.

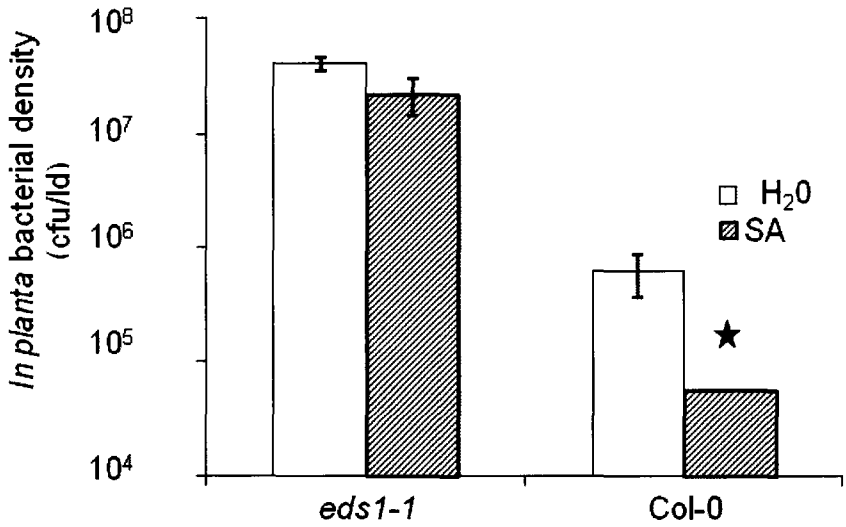
Figure 12. SA application in *iap1-1* and Col-0. Leaves of 5-week-old plants were inoculated with *Pst* at 5 or 24 hours post SA infiltration (hpSAi with 0.1 mM). *In planta* bacterial levels (cfu/ld) were monitored 3 days after *Pst* inoculation (SA, *Pst*) and compared to control treatments (H_2O, *Pst*), where water infiltration was followed by inoculation with *Pst* 24 h later. Stars indicate a significant decrease in bacterial density in SA-treated plants versus the corresponding water-treated plants (Student's t test, $P<0.05$). The mean ± SD of three replicate samples is shown. Experiments where *Pst* was inoculated 5 hours post SA infiltration were repeated twice with similar results.



SA infiltration does not *rescue the eds1-1 ARR defect*

Since *EDS1* is required for ARR and SA accumulation, and intercellular SA infiltration partially rescued the *iap1-1* ARR defect, a similar experiment was performed with *eds1-1*. Addition of SA to the intercellular space of *eds1-1* prior to inoculation with *Pst* resulted in a ~ 2-fold reduction (not statistically significant) in bacterial density compared to water-infiltrated control plants, however these plants still supported high levels of *Pst* (~$2x10^7$ cfu/ld) (Figure 13). It is interesting to note that in 1 of 2 other experiments, there was a statistically significant reduction in *Pst* growth in SA-infiltrated *eds1-1* plants, however *Pst* levels remained high (10^7 cfu/ld). Thus although addition of SA to the intercellular space of *eds1-1* plants reduced *Pst* levels by 2-fold, *Pst* levels were still above 10^7 cfu/ld indicating that the ARR response was not restored in *eds1-1* by exogenous intercellular SA.

Figure 13. SA application in *eds1-1* and Col-0. *eds1-1* and Col-0 plants (5 wpg) were infiltrated with water (H, *Pst*-5) or SA (0.1mM) (SA, *Pst*-5), followed by inoculation with *Pst* (10^6 cfu/ml) 5 h later as in Figure 16. The mean ± SD of three replicate samples is shown. Stars indicate a significant decrease in bacterial density (Student's t test, $P<0.05$). This experiment was repeated twice with similar results.



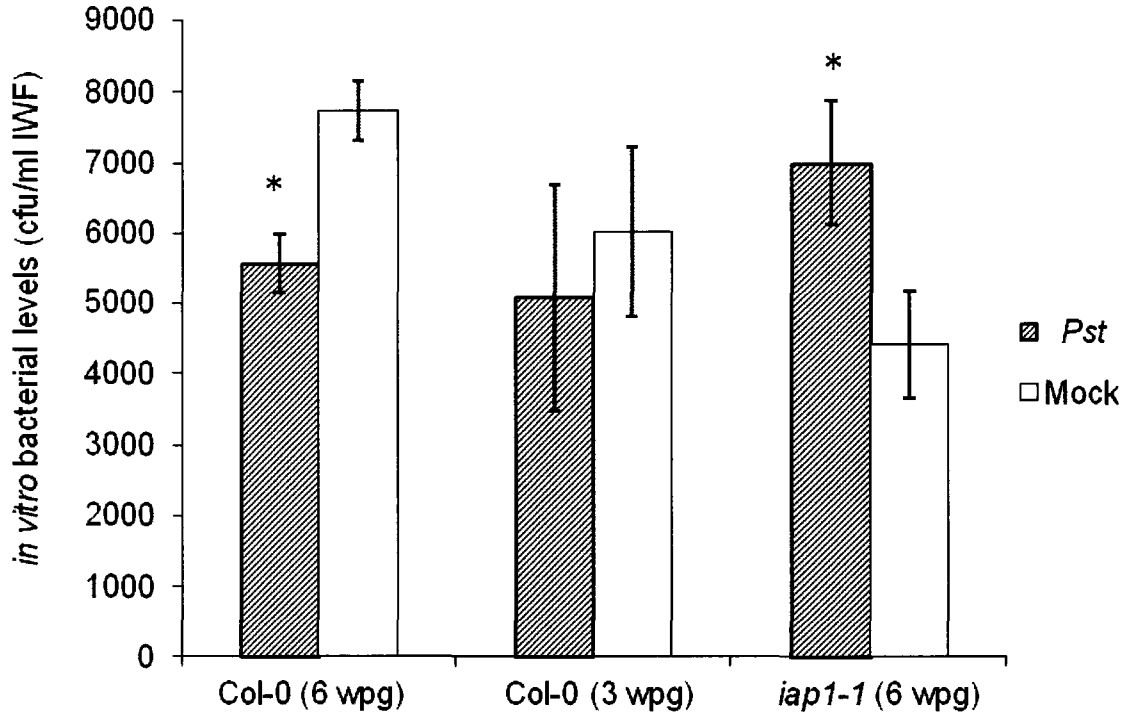
Detection of Anti-Microbial Activity in Intercellular Washing Fluids in iap1-1 compared to Col-0

One of the final steps in the ARR pathway that leads to resistance is thought to be the accumulation of SA in the intercellular space, where it is thought to act as an anti-microbial agent (Cameron and Zaton, 2004). Since the SA rescue experiments mentioned above suggest that ARR is partially rescued in *iap1-1* by the infiltration of SA into the intercellular space, it is possible that SA is not accumulating in the intercellular space in *iap1-1.* Therefore if SA is responsible for intercellular anti-microbial activity this should be reduced in *iap1-1*. An *in vitro* assay was done to measure anti-microbial activity in intercellular washing fluids (IWFs) that represent the intercellular space. IWFs were collected from mature plants 24 hpi and were incubated with a liquid culture of *Pst* for one hour and plated on KB plates to determine bacterial growth.

An ARR assay revealed that bacterial density in Col-0 was 10^6 cfu/ld suggesting it was displaying ARR while *iap1-1* had a high bacterial density at ~10^7 cfu/ld. IWFs from the wild type control, (Col-0) supported significantly less *Pst* than IWFs from mock-inoculated Col-0 plants (Student's t test, $P<0.05$), illustrating the presence of anti-microbial activity in ARR-induced Col-0 plants (Figure 14). Young Col-0 plants (3 wpg) did not display ARR and no difference in bacterial growth was observed in IWFs obtained from plants that were inoculated with virulent *Pst* and plants that were mock-inoculated which was similar to what was observed in *iap1-1*. As a decrease in *Pst* growth was not observed in IWFs

collected from *iap1-1* plants inoculated with virulent *Pst* versus the mock-inoculated control (Figure 14), this suggested that anti-microbial activity is reduced or absent in *iap1-1* and *IAP1* is required to produce anti-microbial activity in the ARR pathway. When this experiment was repeated at 12 or 16 hpi, results were too varied to see a definitive decrease in bacterial growth, in mock versus ARR displaying plants. We hypothesize that the plants did not have sufficient time to accumulate SA to high enough levels to be able to retrieve it from the leaves by centrifugation. It has been observed that IWFs collected from *iap1-1* inoculated with virulent *Pst* actually support higher bacterial growth than mock-inoculated controls. This phenomenon can be explained by hypothesizing that the original bacteria that were first inoculated into the plant manipulated the environment in the intercellular space to promote growth. When IWFs from those plants were then plated with subsequent bacteria, *Pst* growth was enhanced.

Figure 14: Anti-microbial activity in intercellular washing fluids collected from ARR-displaying leaves. Anti-microbial assays were performed with IWFs collected from *iap1-1* plants either mock-inoculated or inoculated with virulent (10^6cfu/ml) *Pst* and compared with wild type Col-0 samples similarly inoculated. IWFs were collected 24 hpi challenge from young plants (3wpg) and mature plants (6wpg) and then incubated for one hour with virulent *Pst*, in liquid culture then plated. The mean ± SD of three replicate samples is shown. Stars represent a significant decrease in bacterial growth in the presence of IWFs from plants inoculated with *Pst* versus mock-inoculated plants (Student's t test $P<0.05$). This experiment was repeated with similar results.

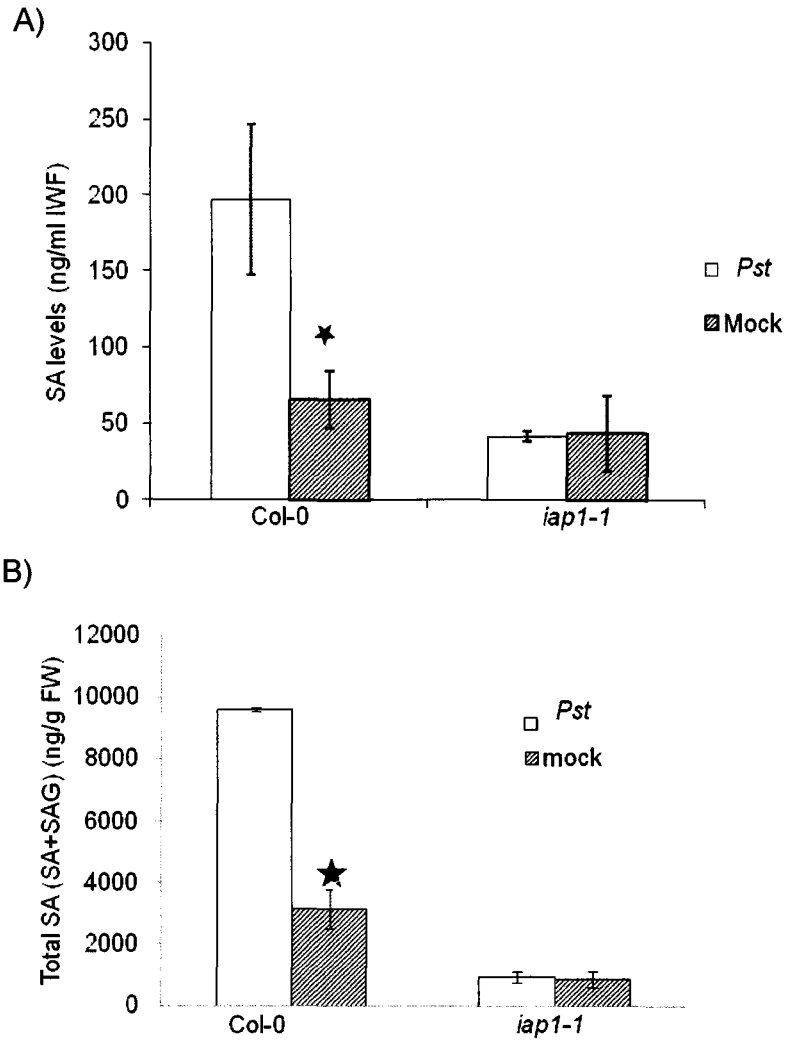


SA accumulation is reduced in mature iap1-1 in response to Pst

The ARR–defective phenotype of *iap1-1* was partially rescued by intercellular SA infiltration. This suggests that *IAP1* lies upstream of intercellular SA accumulation in the ARR pathway which lead to the prediction that *iap1-1* plants will accumulate little intercellular SA compared to wild type plants. To address this question, intercellular washing fluids (IWFs) were collected from mature Col-0 and *iap1-1* plants at 24 hpi with 10 mM $MgCl_2$ or inoculation with *Pst* (10^6 cfu/ml). This time point was chosen as previous work demonstrated that intercellular SA levels increase in IWFs by 24 hpi (Cameron and Zaton, 2004). GC Mass Spectrometry analysis (Schmelz et al., 2004) was employed to measure free SA levels in IWFs collected from ARR-competent Col-0 (*Pst* ~10^5 cfu/ld at 3 dpi) and ARR-incompetent *iap1-1* plants (*Pst* ~10^7 cfu/ld at 3 dpi). IWFs collected from Col-0 leaves inoculated with *Pst* accumulated ~190 ng ml^{-1} SA compared to ~60 ng ml^{-1} in IWFs collected from mock-inoculated leaves (Figure 19a). No significant difference (Student's t test) in intercellular SA accumulation was observed in IWFs collected from *iap1-1* leaves either mock-inoculated or inoculated with *Pst* (~50 ng ml^{-1}) (Figure 19a). These data demonstrate that *iap1-1* does not accumulate intercellular SA in response to *Pst* and corroborates the idea that *IAP1* is upstream of intercellular SA accumulation in the ARR pathway. To determine if *iap1-1* is unable to transport SA to the intercellular space or unable to accumulate SA, intracellular SA was measured in leaf tissue (after IWF removal) during ARR. *iap1-1* accumulated less free SA

and total SA (free SA plus stored SA β-glucoside (SAG)) at ~ 140 and 860 ng g FW^{-1} respectively compared to ~360 and 8640 ng g FW^{-1}, observed in Col-0 controls, respectively (Figure 15b). These results suggest that the *iap1-1* mutation is disrupting the accumulation of SA and not the transport of SA to the intercellular space.

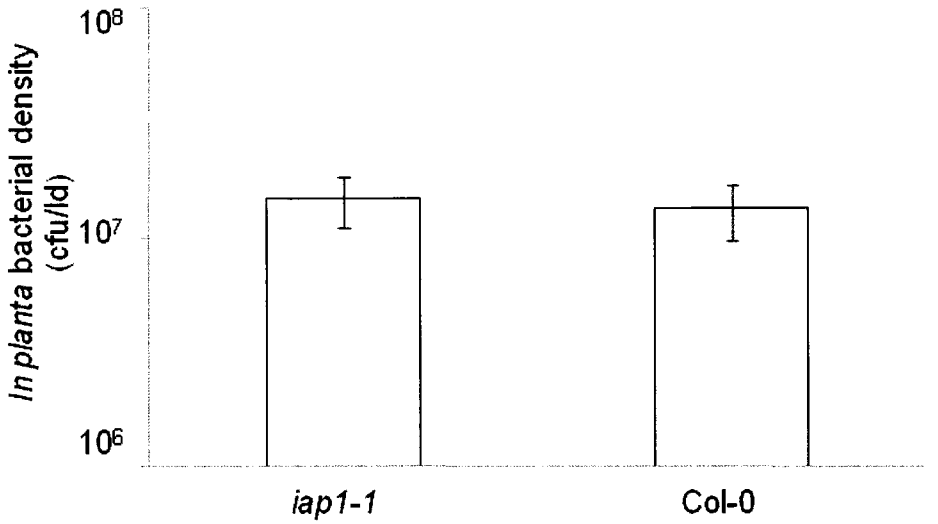
Figure 15. Intercellular and intracellular SA in mature (6 wpg) *iap1-1* and Col-0. IWFs were collected from mature leaves 24 hpi with *Pst* and controls that were mock-inoculated ($MgCl_2$). SA levels were determined in both IWFs A) and leaf tissue with IWFs removed B) and are presented as ng/ml of IWF collected and ng/g FW. Stars indicate a significant decrease in SA (Student's t test $P<0.05$). The mean ±SD of three replicate samples is shown. The IWF intercellular SA measurement was repeated twice and intracellular SA measurement was repeated once with similar results.



iap1-1 is not compromised in basal resistance

Since it was hypothesized that *iap1-1* mutants may not be accumulating SA in mature plants during ARR we also wished to determine if *IAP1* was important for disease resistance in young plants. Young (3 wpg) *iap1-1* and Col-0 controls, were inoculated with virulent *Pst* (10^6 cfu/ml) and bacterial growth was monitored 3 dpi (Figure 16). Virulent *Pst* is able to suppress the basal resistance pathway therefore *iap1-1* would be more susceptible if basal resistance were compromised. Even though basal resistance is suppressed by *Pst* the entire pathway is not completely abolished as wild type Col-0 still support less bacterial growth than *NahG* which accumulates little SA and displays increased susceptibility (>10^7 cfu/ld in *NahG*). Bacterial growth in young *iap1-1* and Col-0 was not significantly different (Student's t test <0.05) indicating that basal resistance is not affected by the *iap1-1* mutation.

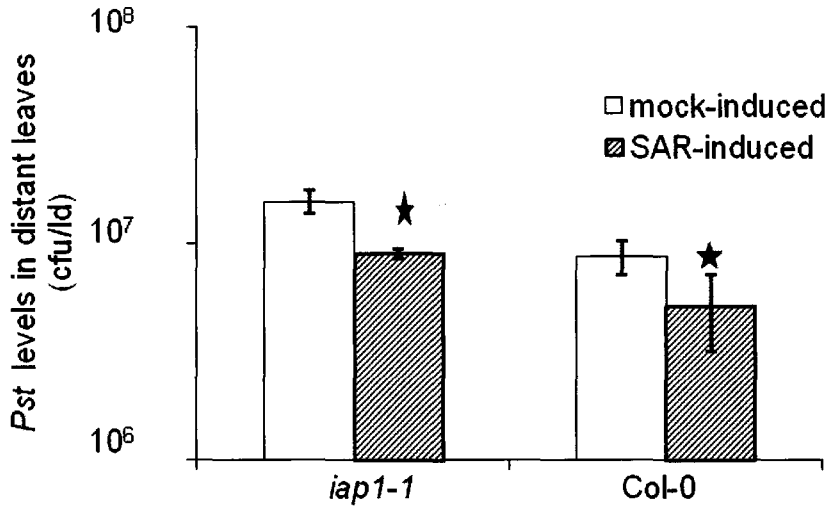
Figure 16. Basal resistance in *iap1-1*. Col-0 and *iap1-1* were inoculated with 10^6 cfu/ml *Pst* at 3 wpg. *In planta* bacterial levels were monitored 3 dpi and are presented as the mean ± SD of three samples. This experiment was repeated three times with similar results.



iap1-1 displays SAR

Since mature (6wpg) *iap1-1*accumulate little SA, and SAR is another pathogen defense pathway that requires SA, *iap1-1* was tested for its ability to display SAR to determine if *IAP1* is involved in the SAR pathway. SAR is a broad spectrum form of resistance that is induced by a local infection resulting in resistance in distant tissues to normally virulent pathogens. Young (3 wpg) *iap1-1* mutants and Col-0 controls were either induced for SAR with *Pst(avrRpt2)* (10^6 cfu/ml) or were mock-inoculated with 10 mM $MgCl_2$ on two lower leaves. Two dpi, distant leaves were challenged with virulent *Pst* (10^5cfu/ml). A significant, (Student's t test $P<0.05$) decrease in bacterial growth in distant leaves was observed in wild type Col-0 that had been induced for SAR as compared with mock-induced control plants (Figure 17). *iap1-1* plants induced for SAR with *Pst(avrRpt2)* supported approximately two-fold less bacterial growth compared with mock-inoculated controls in distant leaves challenged with virulent *Pst*, while wild type plants displayed an approximate four-fold difference. Although Col-0 displayed a greater decrease in bacterial growth between the two treatments, there was also a proportionally larger standard deviation. Overall, the decrease in bacterial growth was modest, a robust SAR response results in greater than ten-fold difference in bacterial growth. A modest SAR response was also observed in replicate experiments. These results suggest that the SAR pathway is still functional in *iap1-1* however these results must be repeated.

Figure 17: SAR in the *iap1-1* mutant. Mutant *iap1-1* plants and Col-0 (3wpg) were inoculated with *Pst(avrRpt2)* (10^6 cfu/ml) or mock-inoculated with 10 mM $MgCl_2$ in two lower leaves followed by inoculation with virulent *Pst* (10^5 cfu/ml) 3 days later in distant leaves. *In planta* bacterial density was determined three dpi and is presented as the mean of three samples ± SD. Stars denote a significant decrease in bacterial growth between SAR-induced and mock-induced controls using the Student's t test ($P<0.05$). This experiment was repeated three times with similar results.



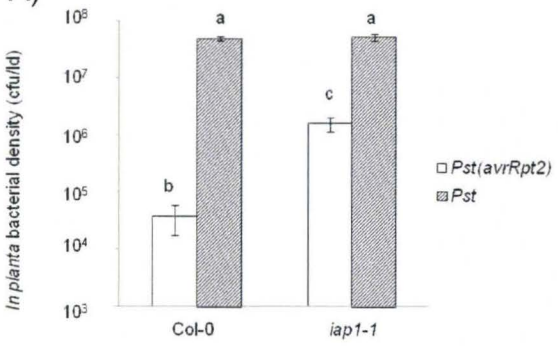
iap1-1 is partially compromised in R gene-mediated resistance to Pst(avrRpt2)

As *iap1-1* accumulates little SA during ARR (Figure 18), and SA is required for the R gene-mediated resistance pathway, *IAP1*'s ability to contribute to R gene-mediated defense was investigated. In wild type plants *Pst (avrRpt2)* is normally avirulent as the R gene product RPS2 detects the presence of the virulence effector avrRpt2 and initiates the R gene-mediated resistance response (Kunkel et al., 1993; Yu et al., 1993). Young (3 wpg) *iap1-1* and Col-0 were inoculated with 10^6 cfu/ml *Pst* and *Pst(avrRpt2).* Both *iap1-1* and Col-0 supported high levels of virulent *Pst* (~ 10^7 cfu/ml) (Figure 18a). *Pst(avrRpt2)* levels were almost 5-fold higher in young *iap1-1* versus Col-0. *iap1-1 Pst(avrRpt2)* levels however were almost 17-fold lower than virulent *Pst* levels. These results show that there is a partial decrease in R gene-mediated resistance in *iap1-1*, suggesting that *IAP1* is required.

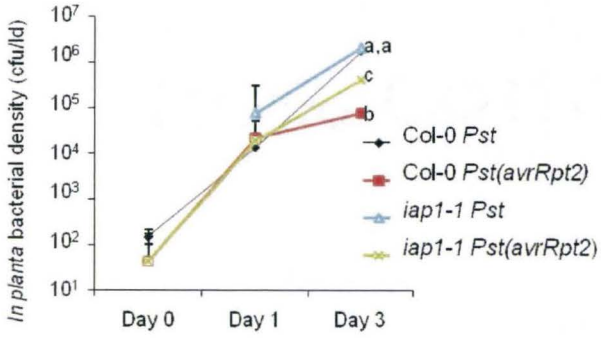
Bacterial growth was also monitored over three days to determine when the R gene-mediated resistance defect in *iap1-1* is first observed. Bacterial growth in Col-0 and *iap1-1* was similar at 0 and 1 dpi, demonstrating that the number of bacteria infiltrated and *Pst(avrRpt2)* growth over the first day is similar in both. At 3 dpi, Col-0 plants displayed R gene-mediated resistance, whereas *Pst(avrRpt2)* levels were significantly (over 5-fold) higher in *iap1-1* (anova, $P<0.0001$) (Figure 18b). These results suggest that R gene-mediated resistance to *Pst(avrRpt2)* is partially affected by the *iap1-1* mutation.

Figure 18. R gene-mediated resistance to *Pst(avrRpt2)* in *iap1-1*. A) Col-0, and *iap1-1* were inoculated with 10^6 cfu/ml *Pst* and *Pst(avrRpt2)* at 3 wpg. *In planta* bacterial density was monitored 3 dpi and is presented as the mean ± SD of three samples. Letters indicate a significant decrease in bacterial density in plants inoculated with *Pst(avrRpt2)* compared to *Pst* inoculated plants (anova, P<0.0001). This experiment was repeated three times with similar results. B) *In planta* bacterial growth in *iap1-1* and Col-0 plants (3 wpg) was measured over 3 dpi (10^6 cfu/ml *Pst* and *Pst(avrRpt2))* and is presented as the mean of three samples ± SD. Letters indicate a significant decrease in bacterial density in plants inoculated with *Pst(avrRpt2)* compared to *Pst* inoculated plants (anova, P<0.0001). This experiment was repeated three times with similar results.

A)



B)



iap1-1 is partially compromised in R gene-mediated resistance to Pst(avrRps4)

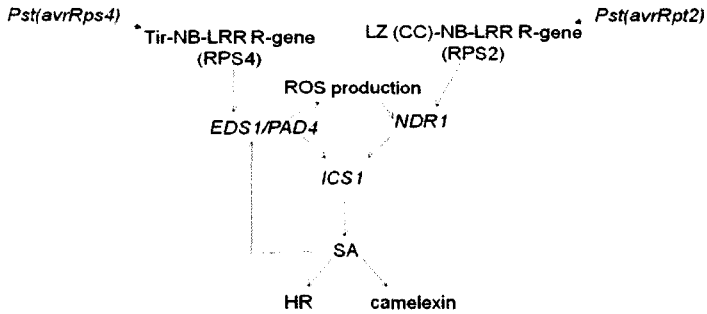
As shown above, *iap1-1* displays a partial R gene-mediated resistance response to *Pst(avrRpt2)*. Since *iap1-1*'s response to *Pst(avrRpt2)* is partially disrupted, *IAP1* is probably required for some aspect of the NDR1 branch of the R gene-mediated resistance pathway. Considering that *iap1-1* accumulates little SA this may be the cause of the partial disruption in R gene-mediated resistance in *iap1-1*. NDR1 is not the only regulator that can initiate an R gene-mediated resistance response that requires SA. Many of the R genes that do not signal through NDR1 use the EDS1 pathway (Figure 19a). Studying the response of *iap1-1* to both *Pst* containing *avrRpt2* (NDR1-dependent pathway) and *avrRps4* (EDS1-dependent pathway) will reveal if *iap1-1* is partially defective for two of the major branches of the R gene-mediated resistance pathway (which may suggest that *IAP1* has a role in HR which is common to both pathways), or if the partial defect is specific to the NDR1 or EDS1 pathway. Young (3 wpg) *iap1-1* and Col-0 were inoculated with 10^6 cfu/ml *Pst* and *Pst(avrRst4)*. Avirulent *Pst(Rps4)* growth is compared to virulent *Pst* growth because the avirulence product avrRps4 induces R gene-mediated resistance in *Arabidopsis* while virulent *Pst* does not. Therefore by comparing bacterial growth in plants that were innoculated with *Pst(avrRps4)* and plants that were inoculated with *Pst* it is possible to observe the difference in bacterial growth during R gene-mediated resistance and other forms of resistance such as basal resistance. Both *iap1-1* and Col-0 supported high levels of virulent *Pst* (~ 10^7 cfu/ml) (Figure 19b).

Avirulent *Pst(avrRps4)* levels in young *iap1-1* were almost 20-fold higher than in Col-0, but remained almost 30-fold lower than virulent *Pst* levels indicating that R gene-mediated resistance to *Pst(avrRps4)* is partially affected by the *iap1-1* mutation.

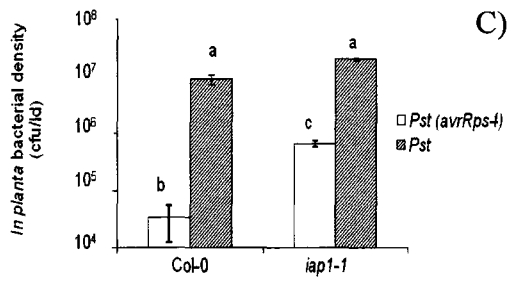
Bacterial growth was also monitored over three days to determine when the R gene-mediated resistance defect in *iap1-1* is first observed. Bacterial growth in Col-0 and *iap1-1* was similar at 1 and 2 dpi, demonstrating that the number of bacteria infiltrated and *Pst(avrRps4)* growth over the first two days is similar in both. At 3 dpi, Col-0 plants displayed R gene-mediated resistance, whereas *Pst(avrRps4)* levels were almost 30-fold higher in *iap1-1* (Figure 19c), suggesting that IAP1 is required for R gene-mediated resistance.

Figure 19. R gene-mediated resistance to *Pst(avrRps4)* in *iap1-1*. A) Two R gene-mediated resistance pathways tested in *iap1-1*. B) Col-0, and *iap1-1* were inoculated with 10^6 cfu/ml *Pst* and *Pst(avrRps4)* at 3 wpg. *In planta* bacterial density was monitored 3 dpi and is presented as the mean ± SD of three samples. Letters indicate a significant decrease in bacterial density (anova $P<0.0001$). This experiment was repeated twice with similar results. C) *In planta* bacterial growth in *iap1-1* and Col-0 plants (3 wpg) was measured over 3 dpi (10^6 cfu/ml *Pst* and *Pst(avrRps4))* and is presented as the mean of three samples ± SD. By day three *Pst(avrRps4)* bacterial density in Col-0 is significantly lower than *iap1-1* (anova $P<0.0001$). This experiment was repeated with similar results.

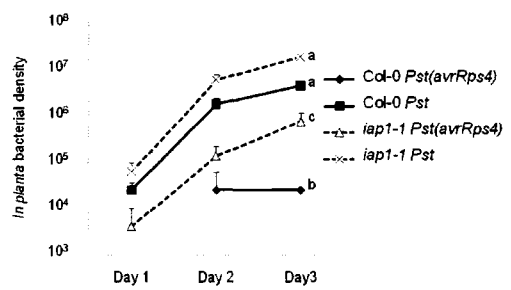
A)



B)



C)



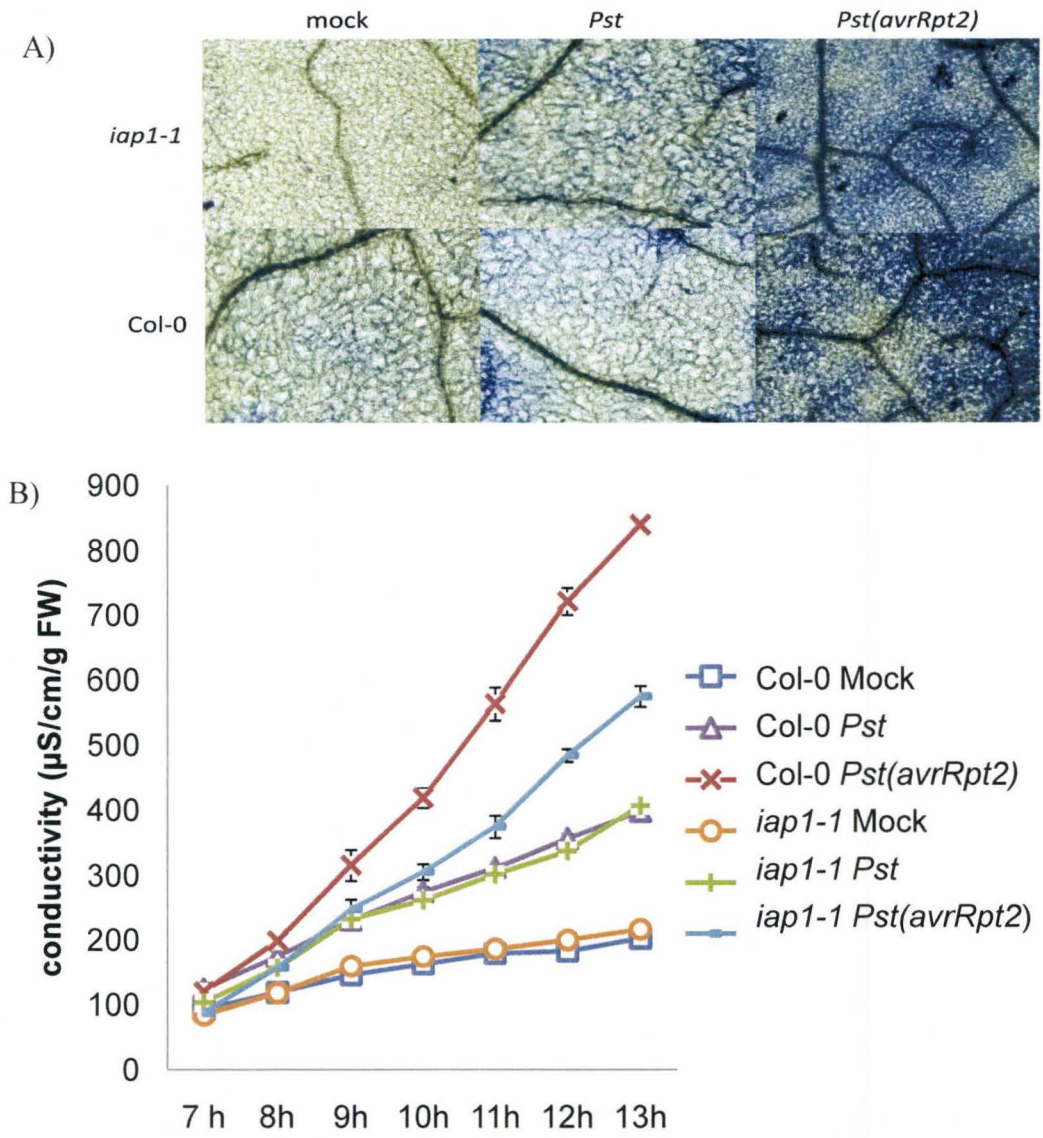
iap1-1 mutants display a less robust HR compared with wild type

As *iap1-1* displays a partial deficiency in R gene-mediated resistance to two separate effectors (*avrRpt2, avrRps4*) which activate resistance through two different regulators (NDR1, EDS1) *iap1-1* mutants were tested for the ability to display HR because it is downstream of both regulators and common to both pathways. Furthermore, in many cases HR is the end result of the R gene-mediated resistance response. Therefore it was expected that *iap1-1* would not display HR. Twelve leaves were collected from Col-0 and *iap1-1* plants 24 hpi with *Pst* or *Pst(avrRpt2)* (10^7 cfu/ml) along with mock-inoculated (10 mM $MgCl_2$) controls and stained with trypan blue to detect cell death (Figure 20a). Trypan blue is a diazo dye that stains dead cells blue. It cannot pass through intact cell membranes of live cells which makes it a useful tool to detect HR-mediated cell death that would be expected if a plant had activated the HR response. Visual analysis revealed little cell death in mock-inoculated and *Pst*-inoculated plants. More significant cell death was observed in *Pst(avrRpt2)*-inoculated plants. *Pst(avrRpt2)*-inoculated *iap1-1* plants had a similar amount of cell death to that in wild type Col-0 plants (Figure 20a).

During the HR there is a steady efflux of ions (mostly hydroxide and potassium ions) from cells as death occurs making it possible to quantify ion leakage as a measure of the HR response (Keppler and Novacky 1986). Col-0 and *iap1-1* plants were inoculated with *Pst* or *Pst(avrRpt2)* (10^7 cfu/ml) along with mock-inoculated (10 mM $MgCl_2$) controls. Loss of ions caused by plasma

membrane damage during the HR response was measured by monitoring conductivity hourly from 7 to 13 hpi. Ion leakage in Col-0 was ~4 fold higher in *Pst(avrRpt2)*-inoculated plants versus mock-inoculated controls while a 2.7-fold increase in ion leakage was observed in *Pst(avrRpt2)*-inoculated *iap1-1* in comparison with mock-inoculated *iap1-1* (Figure 20b). *iap1-1* mutants are capable of an HR response during R gene-mediated resistance however these results suggest that it is a less robust response compared with wild type controls.

Figure 20: HR in *iap1-1* and Col-0. A) Col-0 and *iap1-1* were inoculated with 10^7 cfu/ml *Pst* or *Pst(avrRpt2)* or were mock-inoculated at 4 wpg. Leaves were stained with trypan blue at 24 hpi to reveal cell death. This experiment was repeated three times with similar results. B) Ion leakage was monitored by conductivity over 7 to 13 hpi and is presented as the mean ± SD of three samples. This experiment was repeated twice with similar results.

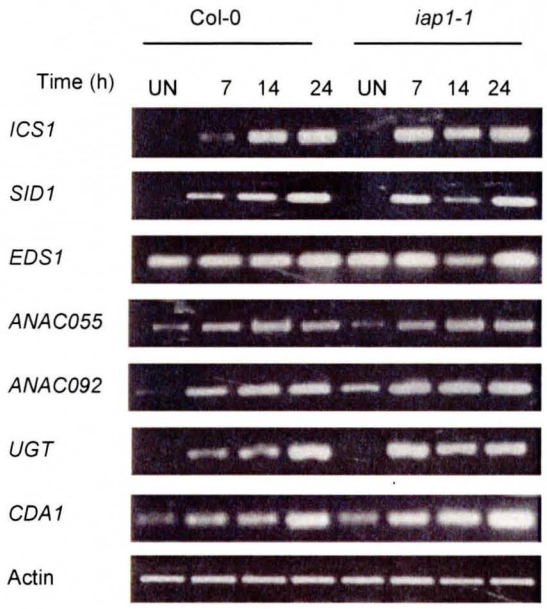


ARR gene expression analysis in Col-0 and iap1-1

Mature (6wpg) *iap1-1* heterozygous mutants support higher bacterial densities than wild type but lower bacterial densities than homozygous *iap1-1* mutants when inoculated with *Pst*. This evidence suggests the *iap1-1* mutation is semi-dominant. A mutation in a positive regulator could cause a semi-dominant phenotype as the greater the expression of the regulator, the greater the expression of ARR genes. Therefore the semi-dominant nature of *IAP1* led to the hypothesis that *IAP1* might act as a positive regulator of downstream genes in the ARR pathway. To test this hypothesis, reverse transcriptase PCR (RT-PCR) was used to determine if SA-associated genes (*ICS1, SID1, EDS1*) and ARR genes (*ANAC055, ANAC092, CDA1, UGT85A1)* previously identified using microarray analysis (Carviel et al., 2009), were expressed differentially in *iap1-1* compared to Col-0 which would suggest that *IAP1* is upstream in the ARR pathway. Leaves were collected after inoculation with *Pst* (10^6 cfu/ml, 7 to 24 hpi) or left untreated. Expression of *ANAC055, ANAC092, CDA1*, and *UGT85A1*, *ICS1, SID1*, and *EDS1* was similar in *iap1-1* and Col-0 (Figure 21), suggesting that *IAP1* is not upstream of these genes in the ARR pathway. *EDS1* was constitutively expressed in untreated leaves and its expression was not increased in response to virulent *Pst* as observed previously (Falk et al., 1999). It is interesting to note that *ICS1*, the penultimate enzyme in the chorismate biosynthesis pathway (Wildermuth et al., 2001) and *SID1*, a putative chloroplast SA transporter (Nawrath et al., 2002) were expressed early at 7 and 14 hpi

(Figure 21), but these genes were not identified in the microarray experiment which represents ARR gene expression at 12 hpi. The microarray experiment was performed in growth chambers without added humidity in the winter (60% humidity), while all other experiments presented here were performed in chambers with added humidity (humidity maintained between 70-85%). *Pst* growth is enhanced under conditions of higher humidity (Agrios, 2005) therefore in higher humidity chambers, *Pst* would begin the infection process quickly, leading to earlier initiation of ARR, and this may explain why *SID2* and *SID1* (Figure 21) were expressed at earlier time points during ARR.

Figure 21. ARR gene expression analysis in *iap1-1* and Col-0. RT-PCR analysis was performed to monitor *ICS1, SID1, EDS1, ANAC055, ANAC092, CDA1*, and *UGT85A1* gene expression in Col-0 and *iap1-1*. Mature (6 wpg) Col-0 and *iap1-1* leaves were collected from 7 to 24 hpi with *Pst* (10^6 cfu/ml) and untreated controls (UN). *ACTIN* was used as a loading control. Twenty-eight cycles of PCR were used for all primers. This experiment was repeated two additional times with similar results.

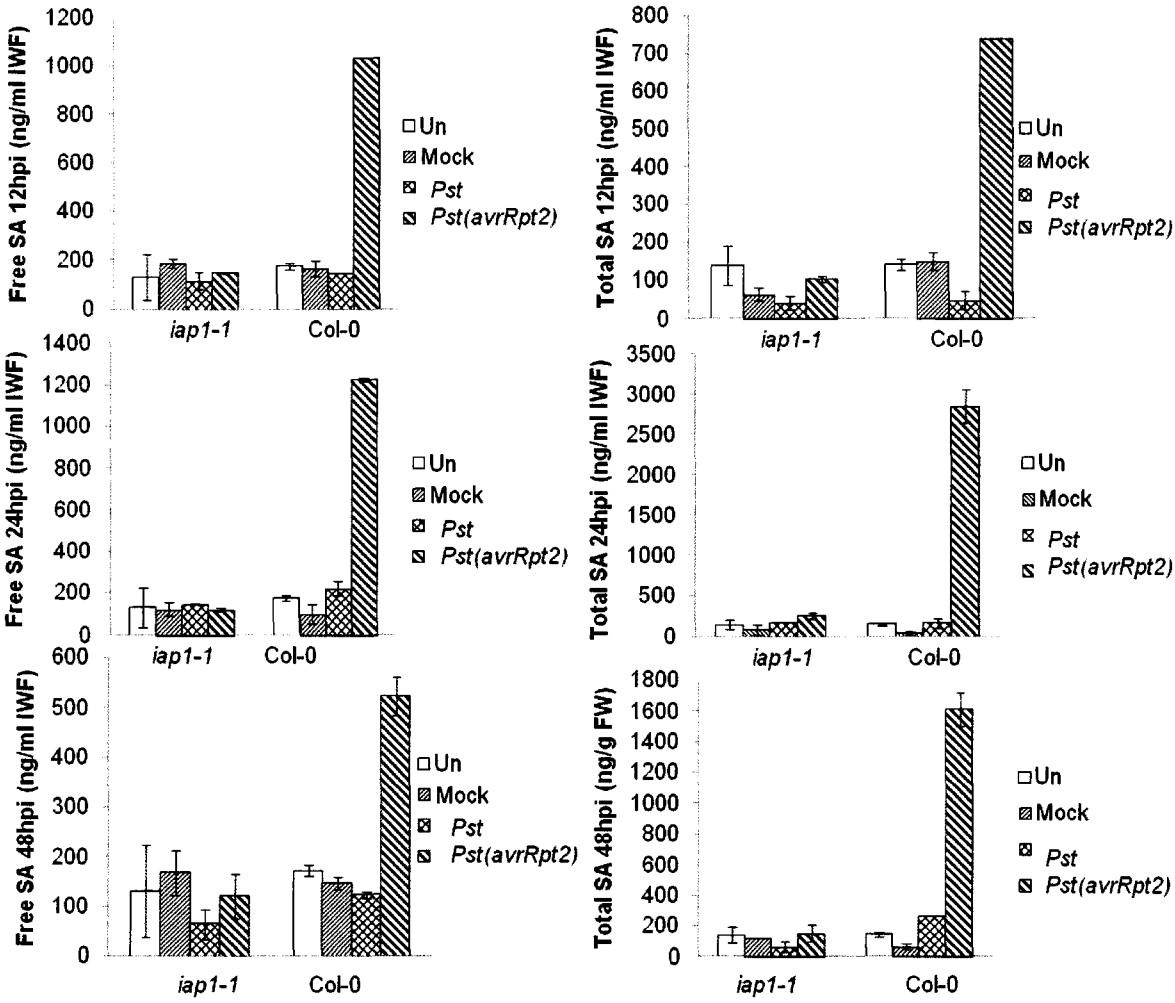


Intercellular SA accumulation is reduced in young iap1-1 plants in response to Pst(avrRpt2)

SA levels in mature *iap1-1* plants were previously shown (Figure 22) to be significantly lower as compared to wild type during ARR. As basal resistance requires SA and seems to be intact in *iap1-1,* it was hypothesized that SA would accumulate normally in young plants. R gene-mediated resistance to *Pst*(*avrRpt2*) and *Pst(avrRps4)* however, is partially disrupted in *iap1-1* suggesting that SA accumulation could be reduced (Carviel and Cameron, in preparation) and it was hypothesized that the *iap1-1* mutation may be disrupting SA accumulation. To determine if young *iap1-1* mutants can accumulate SA, IWFs were collected from young (4 wpg) Col-0 and *iap1-1* plants at 24 hours post mock-inoculation with 10 mM $MgCl_2$ or inoculation with *Pst* or *Pst (avrRpt2)* (10^6 cfu/ml) at 12, 24, and 48 hpi (Figure 22). These time points were chosen as previous work demonstrated that intercellular SA levels increase in IWFs by 24 hpi (Cameron and Zaton, 2004). ADPWH_*lux* which is a non-pathogenic soil bacterium that has been modified to emit a proportional amount of luciferase in the presence of SA (Huang et al., 2006) was employed to measure free SA and total SA (SA plus SAG) as described previously (DeFraia et al., 2008) in IWFs collected from wild type Col-0 and mutant *iap1-1* plants. At 12 hpi both IWFs collected from Col-0 leaves and *iap1-1* leaves accumulated less than 200 ng/ml of SA with the exception of Col-0 leaves that had been inoculated with *Pst(avrRpt2).* IWFs collected from Col-0 leaves that were inoculated with

Pst(avrRpt2) accumulated just under 1000ng/ml of free SA. Similar results were observed for the accumulation of total SA. At 24 hpi no increase in free SA or total SA accumulation was observed in IWFs collected from *iap1-1* and Col-0, again with the exception of IWFs collected from Col-0 that had been inoculated with *Pst(avrRpt2)*. Free SA in IWFs that were collected from Col-0 leaves that were inoculated with *Pst(avrRpt2)* increased to ~1200 ng/ml IWF and total SA increased to ~2700 ng/ml IWF. At 48 hpi the only change that was observed was in IWFs collected from Col-0 leaves inoculated with *Pst*(a*vrRpt2*) which accumulated ~500 ng/ml IWF of free SA and ~1600 ng/ml of total IWF SA (Figure 22). This data demonstrates that *iap1-1* accumulates little intercellular SA in response to *Pst or Pst(avrRpt2)* and suggests that *IAP1* lies upstream of intercellular SA accumulation in the R gene-mediated resistance pathway. As both wild type and *iap1-1* SA accumulation in response to virulent *Pst* was low in comparison with mock-inoculated controls, it was hypothesized that the *Pst* may be suppressing basal resistance-induced intercellular SA accumulation.

Figure 22. Intercellular SA in young (4 wpg) *iap1-1* and Col-0. IWFs were collected from young leaves (4 wpg) at 12, 24, and 48 hpi with *Pst* and *Pst(avrRpt2)* (10^6 cfu/ml) and controls that were mock-inoculated ($MgCl_2$) or left untreated. Free SA and total (free SA plus SAG) levels were determined in IWFs and are presented as ng/ml IWF. The mean ±SD of three replicate samples is shown. This experiment was repeated with similar results.

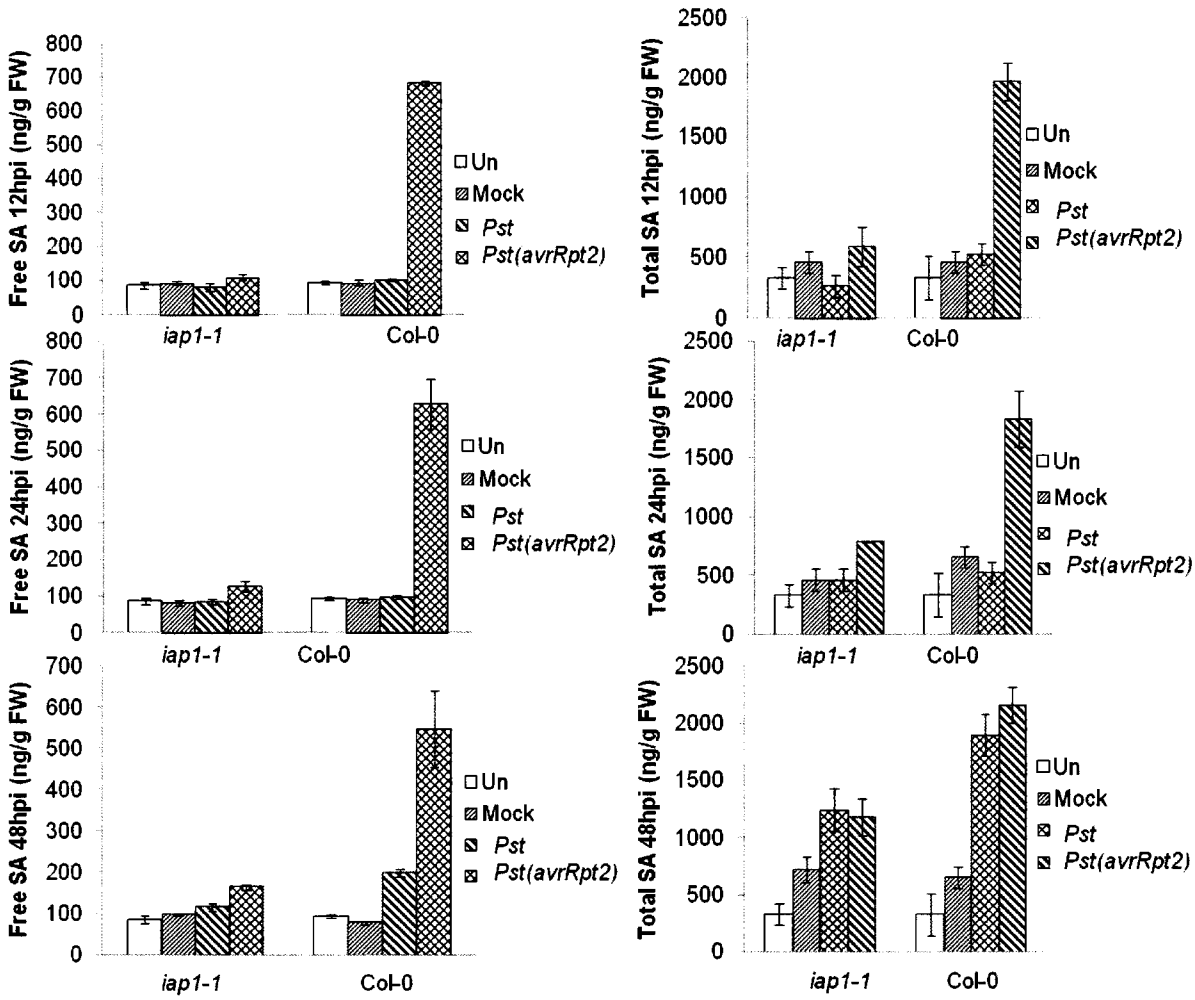


Intracellular SA accumulation is reduced in young iap1-1plants in response to Pst and Pst(AvrRpt2)

Since the *iap1-1* mutation was able to disrupt intercellular SA accumulation, it was hypothesized that intracellular SA accumulation may be disrupted as well. To determine if *iap1-1* also accumulates little intracellular SA during basal and R gene-mediated resistance, SA was measured in leaf tissue (IWFs removed) by incubating ground leaf tissue with the biosensor ADPWH_*lux* (Huang et al., 2006) which produces luciferase in the presence of SA. The amount of luciferase present corresponds with the amount of SA. Leaves were collected from *iap1-1* and Col-0 that were mock-inoculated or inoculated with *Pst* or *Pst*(*avrRpt2*) (10^6 cfu/ml). *iap1-1* accumulated less SA in response to both virulent (*Pst*) and avirulent (*Pst*(*avrRpt2*)) inoculations. In response to *Pst* inoculations at 12 hpi intracellular free SA and total SA did not accumulate in either *iap1-1* or Col-0 as compared with untreated and mock-inoculated controls (~100 ng/g FW and ~500 ng/g FW respectively) (Figure 23). However leaf tissue from *Pst(avrRpt2)* inoculated Col-0 accumulated ~700 ng/g FW of free SA and ~2000 ng/g FW of total SA. At 24 hpi leaf tissue collected from *Pst* inoculated *iap1-1* and Col-0 as well as *Pst(avrRpt2)* inoculated *iap1-1* did not accumulate free SA as compared with untreated and mock-inoculated controls (~100 ng/g FW) (Figure 23). Total SA accumulation did not increase in both *Pst* inoculated *iap1-1* and Col-0 (~500 ng/g FW) (Figure 23). Total SA accumulation in leaf tissue collected from *Pst(avrRpt2)* inoculated leaves increased to ~600 ng/g FW for *iap1-1* and ~1700 ng/g FW for Col-0. By 48 hpi little free SA accumulation

was observed except for leaf tissue collected from *Pst(avrRpt2)* inoculated Col-0 which was ~550 ng/g FW. Total SA accumulation in leaf tissue collected from both *Pst* and *Pst(avrRpt2)* inoculated *iap1-1* increased to ~1200 ng/g FW (Figure 23). Total SA accumulation in leaf tissue collected from both *Pst* and *Pst(avrRpt2)* inoculated Col-0 increased to ~2000 ng/g FW (Figure23). Little SA accumulates in *iap1-1* response to *Pst* and *Pst(avrRpt2)* (Figure 23) until 48 hpi. This observation correlates with previous data that *iap1-1* is able to display a partial R gene-mediated resistance response. This evidence suggests that the *iap1-1* mutation is disrupting intracellular accumulation of SA during both basal resistance and R-gene mediated resistance to *Pst* and *Pst*(*avrRpt2*).

Figure 23. Intracellular SA in young (4 wpg) *iap1-1* and Col-0. IWFs were collected from young leaves (4 wpg) 12, 24, and 48 hpi with *Pst* and *Pst(AvrRpt2)* (10^6 cfu/ml) and controls that were mock-inoculated ($MgCl_2$) or left untreated. SA levels were determined in leaf tissue with IWFs removed and are presented as ng/g FW. The mean ±SD of three replicate samples is shown. This experiment was repeated with similar results.



Chapter 5: Map-based cloning to identify *IAP1*

IAP1 is an important gene in the ARR pathway and is also involved in basal resistance and R gene-mediated resistance. It was therefore of interest to determine the identity of *IAP1*. All of the experiments in this chapter were completed by the author.

A genetic cross between *iap1-1* (Col-0 background) and *Landsberg erecta (Ler)* (wild type for ARR) was performed and produced 36 viable seeds which was sufficient to create the F1 generation. CAPS (Cleaved Amplified Polymorphic Sequences) molecular markers were used to confirm heterozygosity and provided evidence that the F1 plants contained DNA from both Col-0, (from the *iap1-1* mutant,) and *Ler.* CAPS molecular markers take advantage of the presence of single nucleotide polymorphisms (SNPs) between the two ecotypes. For example, after PCR the CAPS molecular marker T6P5-14 would be digested with the restriction enzyme EcoR1 and then would be expected to produce one band on an agarose gel at 1.4 kb if *Ler* DNA was used. If the DNA originated from Col-0 which contains an EcoR1 restriction site in the area of the genome amplified by the marker T6P5-14, then two bands at 1.1 kb and 0.32 kb would be expected. In the case of a heterozygote, all three bands would be expected. Three bands were observed in the F1 generation.

The F1 generation successfully self-fertilized as seeds were produced without further crossing and created the F2 mapping generation. The *iap1-1* mutation is semi-dominant thus high bacterial growth (~10^7 cfu/ld) was observed

in plants homozygous for the mutant *iap1-1* gene when inoculated with *Pst* (10^6 cfu/ml) in an ARR assay, while plants supporting low bacterial growth (~10^5 cfu/ml) were homozygous for the wild type *IAP1* gene. Plants with an intermediate (~10^6 cfu/ml) level of bacterial growth were heterozygous and were discarded. The *iap1-1* phenotype in the F2 mapping population segregated in a 1:2:1 ratio as would be expected of a semi-dominant mutation. The mapping of this population with CAPS markers revealed weak linkage at 2,322,859 base pairs on chromosome two. Coarse mapping was continued using indel molecular markers (Jander et al., 2000) that use insertion and deletion differences between the Col-0 and *Ler* genomes and can be visualized on an agorose gel. Mapping with indel markers revealed a weak linkage on chromosome two at 244,740 base pairs but finer mapping was unable to reveal a closer linkage. Further mapping with indel markers also revealed weak linkage on chromosome one at 746,958, 1,427,727, 1,997,330, and 2,926,057 base pairs. Weak linkage was also observed in chromosomes three and four. The methods used to create the mapping population may be responsible for the inconsistent results. Seeds were pooled from 11 separate *iap1-1* x *Ler* crossing events to create the F1 generation and all F1 progeny were pooled together to create the F2 mapping population. It is possible that unique recombination events happened in each *iap1-1* x *Ler* crossing event and therefore each F1 plant may be slightly genetically different. This would result in a heterogeneous F2 mapping population which would lead to ambiguous results as *IAP1* would map to more than one location.

Additional genetic crosses between *iap1-1* (Col-0 background) and *Ler* were performed and seeds were not pooled. Indel PCR markers were used to confirm heterozygosity in the F1 generation. The F1 generation successfully self-fertilized and produced the F2 mapping generation. Indel molecular markers were used to map *IAP1* to the long arm of chromosome four. A statistically significant number of both wild type ARR displaying individuals containing *Ler* DNA and ARR-defective individuals containing Col-0 DNA, were observed at a marker located at 18,087,180bp on chromosome four. Further mapping suggested that *IAP1* was located between 18,087,180bp and 18,133,423bp on chromosome four. This area contains 16 genes.

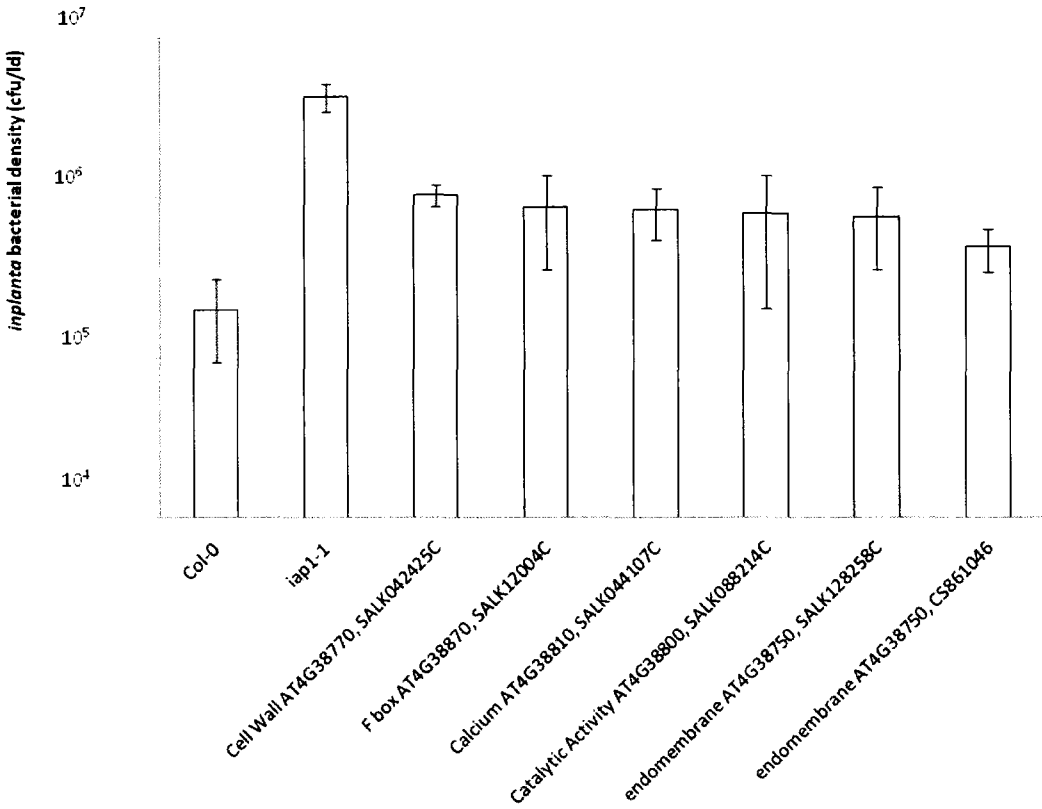
Three candidate genes were chosen and sequenced in *iap1-1*, however no mutations were found. These included a cell wall protein (At4g38770), a protein involved in protein transport (At4g38790), and an auxin responsive protein (At4g38825). The first gene was the auxin responsive gene which was chosen because it was the smallest gene and was used to test the sequencing methods. The coding region of the auxin responsive gene was PCR amplified using Invitrogen Platinum High Fidelity Taq, purified (Norgen DNA purification kit,) and sequenced. The cell wall gene and the protein transport gene were sequenced because these genes were closest to the indel marker that was shown to be statistically closest to *iap1-1* (according to lod scores). The coding sequence was sequenced in the protein transport protein. The coding sequence,

the five prime untranslated region, and the entire intergenic region on the five prime side of the cell wall gene were sequenced.

Plant lines with transfer-DNA (T-DNA) insertions in five of the 16 genes were available from SALK. It was expected that if one of these genes was *IAP1*, interrupting it with a T-DNA should result in a similar ARR phenotype as observed in *iap1-1*. These five T-DNA lines were assayed for ARR and included two alleles of an endomembrane protein (AT4G38750, SALK128258C and CS861046), a cell wall protein (AT4G38770, SALK042425C), a protein with catalytic activity (AT4G38800, SALK088214C), a protein involved in calcium ion binding (AT4G38810, SALK044107C), and an F-box family protein (AT4G38870, SALK12004C). All of these T-DNA insertion lines were verified by other researchers and were documented on The *Arabidopsis* Information Resource (TAIR) website but were not confirmed in the Cameron lab. The cell wall protein T-DNA line displayed a partial ARR phenotype (Figure 24). The milder ARR phenotype (in comparison with *iap1-1*) could be explained by the T-DNA line containing a different allele than *iap1-1* mutants. Sometimes T-DNA lines ordered from SALK are contaminated with heterozygotes which could also explain the modest phenotype. To rule out the presence of heterozygotes, eight plants were screened for ARR individually to ensure similar results. If there were heterozygotes present we would expect to see some plants which support high bacterial growth and some plants which support a medium amount of bacterial growth. Normally during an ARR assay three replicates are collected from a

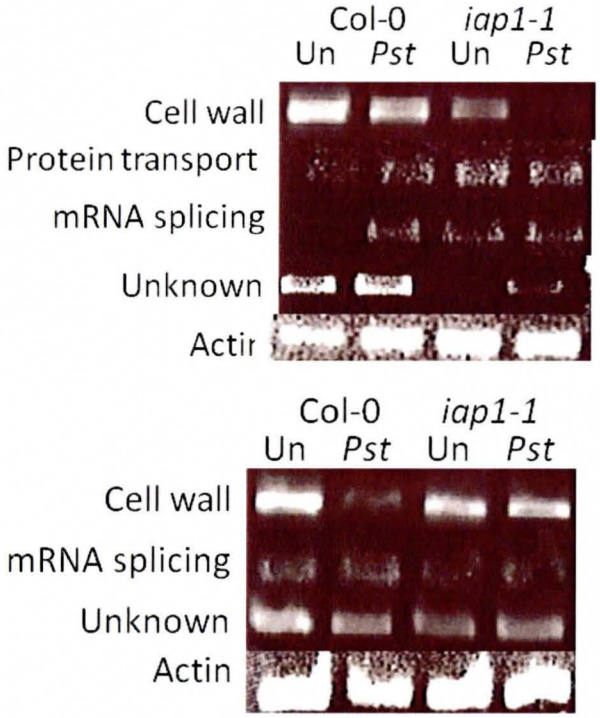
group of nine plants to control for plant to plant variation. To test the T-DNA line for heterozygotes one replicate was collected from each plant. In order to obtain three replicates of each control, three wild type and three *iap1-1* plants were used and one replicate was collected from each. Results were surprisingly variable even for the controls. There does not appear to be heterozygote contamination but this experiment requires repetition with more control plants due to the large variation. There could be many reasons for this variation but it is possible that some individuals were healthier before inoculation and were able to mount a more robust resistance response.

Figure 24. *In planta* bacterial density in T-DNA insertion lines, *iap1-1* and Col-0. T-DNA insertion lines, Col-0, and *iap1-1* were inoculated with 10^6 cfu/ml *Pst* at 6 wpg. *In planta* bacterial levels were monitored at 3 dpi and are presented as the mean ± SD of three samples.



Further mapping subsequently suggested that *IAP1* was located between 18,087,180bp and 18111371bp on chromosome four. This region contains six genes including the cell wall protein, and the endomembrane protein mentioned previously. The endomembrane protein showed no phenotype in the T-DNA insertion line. RT-PCR was used to look for changes in expression in four of the five remaining genes in *iap1-1* compared to Col-0 to try and identify *IAP1*. *IAP1* may be expressed differently in the wild type versus the mutant especially if the mutation is located in the promoter. The fifth gene was less than 100bp long and therefore not a good candidate for RT-PCR. Along with the cell wall protein these genes encode a splice factor (AT4G38780), a protein involved in protein transport (AT4G38790), and an unknown protein (AT4G38760). Expression of the protein transport gene is similar in wild type and *iap1-1* plants (Figure 25). Preliminary results for the other three genes showed expression patterns changing between experiments (Figure 25) and therefore needs to be repeated before conclusions can be made. Expression in the wild type was similar to expression in the mutant in at least one of three experiments which suggests that these genes may not be *IAP1,* especially if the *iap1-1* mutation is in the promoter.

Figure 25. *IAP1* candidate gene expression analysis in *iap1-1* and Col-0. RT-PCR analysis was performed to monitor gene expression in the candidates for *IAP1,* unknown protein (AT4G38760), cell wall protein (AT4G38770), mRNA splicing protein (AT4G38780), protein transport (AT4G38790), and Col-0 and *iap1-1*. Mature (6 wpg) Col-0 and *iap1-1* leaves were collected 24 hpi with *Pst* (10^6 cfu/ml) and untreated controls (UN). *ACTIN* was used as a loading control. 25 cycles of RT-PCR were used for ACTIN, cell wall protein, and protein transport primers while 30 cycles were used for the unknown protein and splicing protein primers.

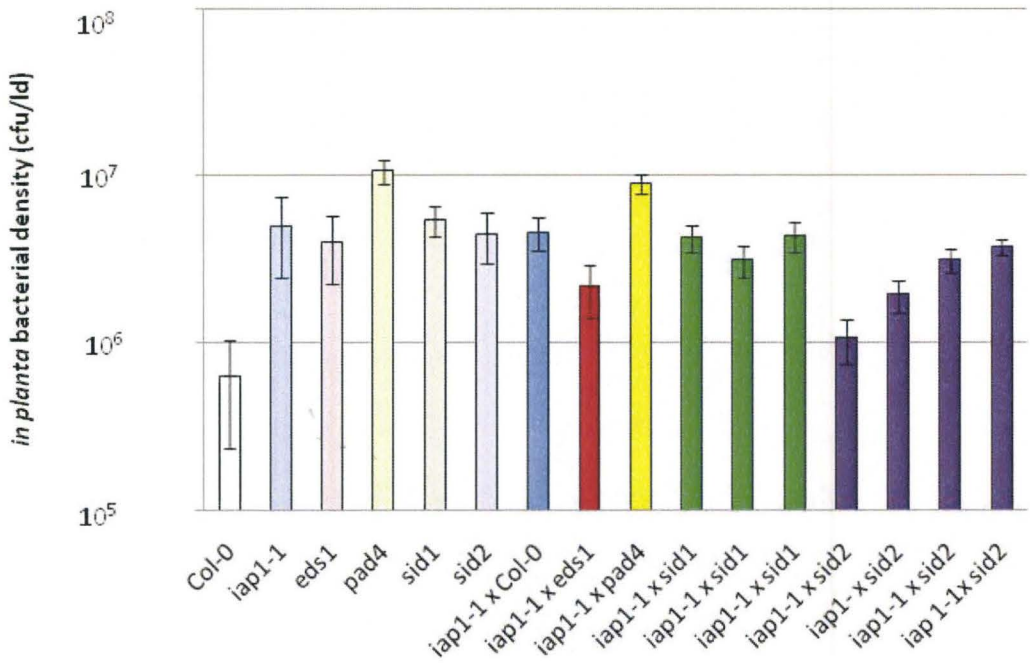


At this point in the mapping process recombination data suggested there might be a problem with some of the individuals. The ARR screen is difficult to use for phenotyping, because it is possible that unhealthy wild type plants appear ARR-defective. Microenvironments such as differences in humidity in different sections of the growth chamber may also affect disease resistance. The virulence of the *Pst* can even differ between experiments due to changes in the weather as the increased humidity of the summer months increases the virulence of *Pst*. The individuals that were producing unexpected results were retested in the F3 generation and it was concluded that they were phenotyped incorrectly in the F2 generation. The challenging nature of the ARR screen has made fine mapping of *IAP1* difficult. The search for *IAP1* has now been expanded from 17,938,268bp to 18,133,423bp because of these problems.

Complementation assays were also used to identify *IAP1*. Four genes have been identified that are required for both ARR and pathogen-induced SA accumulation. This phenotype is also observed in *iap1-1* suggesting that *iap1-1* could be a new allele of one of these four previously identified genes, therefore *iap1-1* was genetically crossed with homozygous *eds1*, *pad4*, *sid1*, or *sid2* mutants. If the *iap1-1* phenotype is the result of a mutation in a different gene, complementation should be observed in the F1 generation. An *iap1-1* heterozygous ARR phenotype would be expected as *IAP1* is semi-dominant and all of the other mutations are recessive. If the *iap1-1* phenotype is the result of a different mutation in *EDS1, PAD4, SID1*, or *SID2* it is expected that the F1

generation of that cross would have an ARR defective phenotype. The F1 generations for each cross were tested for ARR (Figure 26). ARR assays show that ARR was improved when the mutants were crossed with *iap1-1* suggesting that *IAP1* is not one of the four previously identified genes. Unfortunately the partially ARR defective phenotype of the *iap1-1* heterozygote makes it possible to observe only a small improvement in ARR. It is hard to distinguish between homozygous *iap1-1* and heterozygous *iap1-1* as both display increased susceptibility compared to wild type plants. For example there is a very minimal increase in ARR in the *iap1-1/sid1* mutant compared to the *sid1* mutants suggesting that there is still a chance that *iap1-1* is a new allele of *sid1*. Due to the difficult nature of this complementation assay, mapping of *IAP1* will continue along with sequencing of genes near the end of chromosome 4 (17,938,268bp to 18,133,423bp).

Figure 26. Complementation in *iap1-1*. Col-0, *iap1-1, eds1, pad4, sid2*, and *sid1* as well as all genotypes crossed with *iap1-1* were inoculated with 10^6 cfu/ml *Pst* at 6 wpg. *In planta* bacterial density was monitored 3 dpi and is presented as the mean ± SD of three samples. This experiment was repeated with similar results.



Chapter 6: Discussion

This thesis has contributed to our knowledge of ARR. I have identified genes important for ARR, discovered similarities and overlaps with other disease resistance pathways, characterized the ARR gene *IAP1*, and also identified resistance genes that are not required for ARR.

In order to elucidate the ARR pathway candidate genes were tested to determine if they are required for ARR. The EDS1/PAD4 complex is required for pathogen-induced SA accumulation in other *Arabidopsis* disease resistance pathways such as basal resistance and R gene-mediated resistance pathways (Feys et al., 2001; Wiermer et al., 2005). As *PAD4* was previously shown to be required for ARR (Cameron and Zaton, 2004) and SA accumulation is part of the ARR pathway, *EDS1* was hypothesized to be involved in the ARR pathway. High bacterial densities and a lack of ARR in the *eds1-1* mutant confirm its importance in the ARR pathway, revealing another similarity between ARR and R gene-mediated resistance as well as basal resistance. Conversely, the full ARR response observed in both *jar1-1* and *jin1-1* mutants suggests that the JA signaling pathway is not required for the ARR response. Even though RLK (At2g02710) was expressed in an ARR microarray it does not appear to be required for ARR. However, whether cytochrome P450 (*CYP71A13)* is essential for ARR requires further experimentation as the cytochrome P450 T-DNA insertion line was heterozygous, therefore a homozygous cytochrome P450

mutant may be ARR-defective, as has been suggested by other work in the Cameron lab.

The ARR defective mutant *iap1-1* and its semi-dominant nature was identified previously in the Cameron lab. The ARR defective phenotype and semi-dominant nature was confirmed in this thesis. The phenotype of *iap1-1* suggests that it is a loss of function mutation while segregation ratios suggest that it is semi-dominant. Taken together, the characteristics of *iap1-1* hint that IAP1 may be a positive regulator.

Since ARR is thought to result in intercellular SA accumulation, a rescue of the ARR phenotype in *iap1-1* was attempted by infiltrating *iap1-1* with SA prior to challenge with *Pst*. A significant decrease in *in planta* bacterial density was observed when *iap1-1* plants were infiltrated with SA five hours prior to being challenged with *Pst* as compared with sterile water-infiltrated controls. ARR was also shown to be enhanced significantly for Col-0 wild type infiltrated with SA 5 hours prior to inoculation with *Pst* as compared with sterile water-infiltrated controls. These results suggest that SA accumulation may be impaired in the *iap1-1* mutant. Therefore, *IAP1* may be upstream of the accumulation of SA. It was observed, however, that ARR was only partially restored in *iap1-1* in two experiments when compared with Col-0. There was a 24-fold decrease in *in planta* bacterial levels in Col-0 compared to a 4-fold decrease in *iap1-1.* A 9-fold decrease in bacterial levels was observed in Col-0 versus a 2-fold in *iap1-1* in a second experiment. Several explanations could account for this observation. If

iap1-1 is a positive regulator it may control other genes whose products also contribute to ARR. The *iap1-1* mutant may be missing another anti-microbial compound that works in combination with SA, or cell wall modifications may be necessary for a more effective ARR response. It is also possible that the difference in bacterial growth observed between SA-treated Col-0 and *iap1-1* may be explained by ARR working in collaboration with another defense pathway which also requires SA. Therefore intercellular SA infiltration may rescue ARR in *iap1-1* but perhaps not a second, unknown, pathway that complements ARR. If *iap1-1* mutants are defective for another defense pathway that was induced in Col-0 we would expect the difference in enhanced resistance that was observed. The idea that more than one defense pathway can contribute towards a stronger, more effective resistance response is not a new one. It has been noted previously that when SAR and ISR are induced simultaneously, an elevated level of defense is observed (van Wees et al., 2000).

When SA was infiltrated 24 hours prior to challenge, a decrease in bacterial growth was not observed for *iap1-1* or wild type as compared to water-infiltrated controls. These results are not surprising, however, as it has been previously shown that infiltrated SA will move into the cell and SA-infiltrated plants start to lose enhanced resistance approximately 24 hours after infiltration (Cameron and Zaton, 2004). Previous work (Cameron and Zaton, 2004) has shown variation in the amount of SA still in the intercellular space 24 hours after infiltration from 153 ng/ml to ~400 ng/ml. In this experiment it is thought that the

length of time between SA infiltration and *Pst* inoculation was long enough that SA concentrations were no longer high enough in the intercellular space to affect bacterial growth.

Measuring intercellular and intracellular SA in mature (6 wpg) *iap1-1* confirmed the hypothesis that *iap1-1* accumulates little SA in response to virulent *Pst* inoculation and suggests that *IAP1* is upstream of SA accumulation in the ARR pathway. The evidence that *iap1-1* accumulates little SA in the intercellular space also explains the absence of anti-microbial activity in the IWFs as SA is believed to be the ARR intercellular space anti-microbial agent.

In order to determine if *IAP1* is specific to the ARR pathway and contributes to SA accumulation during ARR or if it possibly affects other defense pathways, *iap1-1* mutants were tested for SAR, basal resistance, and R gene-mediated resistance. When compared with mock-inoculated controls a modest decrease in bacterial growth was observed in *iap1-1* during a SAR assay suggesting that *IAP1* is not required for SAR and that it may be specific to ARR. However the SAR response was also modest for Col-0 wild type and there was a large amount of variation in the results suggesting that a robust SAR response was not induced and that these experiments should be repeated. When young (3 wpg) *iap1-1* was tested for basal resistance, high bacterial growth *in planta* and chlorosis in inoculated leaves was observed, but there was no increased susceptibility in comparison with wild type. These results suggest that basal resistance is not affected by the *iap1-1*. R gene-mediated resistance assays

using both *Pst(avrRpt2)* and *Pst(avrRpt4)* resistance revealed that the R gene-mediated resistance pathway was partially disrupted in *iap1-1*, suggesting that *IAP1* may be required for both ARR and R gene-mediated resistance. It is possible that ARR and R gene-mediated resistance pathways share overlapping components.

As SA accumulation is important for both basal and R gene-mediated resistance, plus *iap1-1* accumulates little SA during ARR, it was hypothesized that this SA defect in *iap1-1* may explain the partial R gene-mediated resistance defect as well. SA accumulation was measured in young (4 wpg) *iap1-1* mutants and wild type controls. Intercellular accumulation of SA was observed in response to *Pst(AvrRpt2)* during R gene-mediated resistance, in the same range (153 ng/ml IWF to 400 ng/ml IWF) as previously observed during ARR (Cameron and Zaton, 2004). This evidence suggests that SA may be acting as an antimicrobial agent during both ARR and R gene-mediated resistance (Cameron and Zaton, 2004). *iap1-1* mutants accumulate almost two-fold less intracellular SA and over ten-fold less intercellular SA in response to *Pst(AvrRpt2)* compared to wild type. SA levels in *Pst(avrRpt2)* inoculated *iap1-1* are similar to the background levels of SA observed in untreated and mock-inoculated controls suggesting that *iap1-1* accumulates little SA. This data correlates with the partial disruption of R gene-mediated resistance initiated by two separate R genes (RPS2 and RPS4) in *iap1-1*. These two R gene pathways result in SA accumulation and the HR, but are different in that the RPS2 response is

regulated by NDR1 (Century et al., 1995; 1997) while the RPS4 response is regulated by EDS1 (Aarts et al., 1998). The HR response is also less robust in *iap1-1* mutants when compared with wild type as measured by ion leakage analysis. These results suggest that IAP1 acts in the R gene-mediated resistance pathway downstream of both the EDS1 and NDR1 regulators but upstream of SA accumulation and HR. However since HR cell death is observed in *iap1-1*, it seems likely that IAP1 may be more important for intercellular SA accumulation than HR cell death during R gene-mediated resistance.

It is interesting that the basal resistance response seems to be intact in *iap1-1* (increased susceptibility was not observed in comparison with wild type) even though *iap1-1* mutants have been observed to accumulate almost 500 ng/g FW less intracellular SA in response to pathogen inoculation as compared to wild type at 48 hpi. Two possible explanations for this observation include that smaller amounts of SA are sufficient for a successful basal resistance response as compared with ARR and R gene-mediated resistance. During basal resistance SA is required for NPR1-dependent signaling (Rairdan and Delaney, 2002) versus R gene-mediated resistance, where SA is thought to be necessary for HR, and to potentiate ROS production, (Rairdan and Delaney, 2002) and ARR, where SA is thought to act as an antimicrobial agent (Cameron and Zaton, 2004). Since SA is required for different aspects of each pathway it makes sense that each pathway may require a different amount of SA. A second possibility is that SA accumulation is not required for basal resistance in *iap1-1*. It has been

suggested that the basal resistance response is actually composed of many "layers" of defense and that only some of these layers are SA-dependent (Ham et al., 2007). Examples of SA-independent basal resistance responses include some forms of callose deposition and the strengthening of the cell wall (Ham et al., 2007).

Little intercellular SA accumulation was observed during basal resistance in *iap1-1* and the wild type control. It is possible that the intercellular SA accumulation is a component of basal resistance and *Pst* is able to suppress this part of the response. *Pst* virulence effectors are involved in suppressing the basal resistance response and may contribute to the delayed onset of basal resistance in this system (Kim et al. 2005; Li et al. 2005; Thilmony et al. 2006; Truman et al. 2006). This evidence correlates with our observations that intracellular SA accumulation is delayed (observed at 48 hpi) in the basal resistance response versus R gene-mediated resistance (observed at 12 hpi).

The *iap1-1* mutation reveals similarities and an overlap between three disease resistance pathways as *IAP1* appears to be required for SA accumulation during basal resistance, R gene-mediated resistance, and ARR. *IAP1* is required for intracellular SA accumulation during basal resistance as *iap1-1* accumulates less SA compared to wild type (anova, P=0.0228 at 24 hpi in a replicate experiment). *IAP1* is required for intracellular and intercellular SA accumulation during R gene-mediated resistance and ARR. *IAP1* is also required for a robust HR. Some SA accumulation is observed in *iap1-1* during

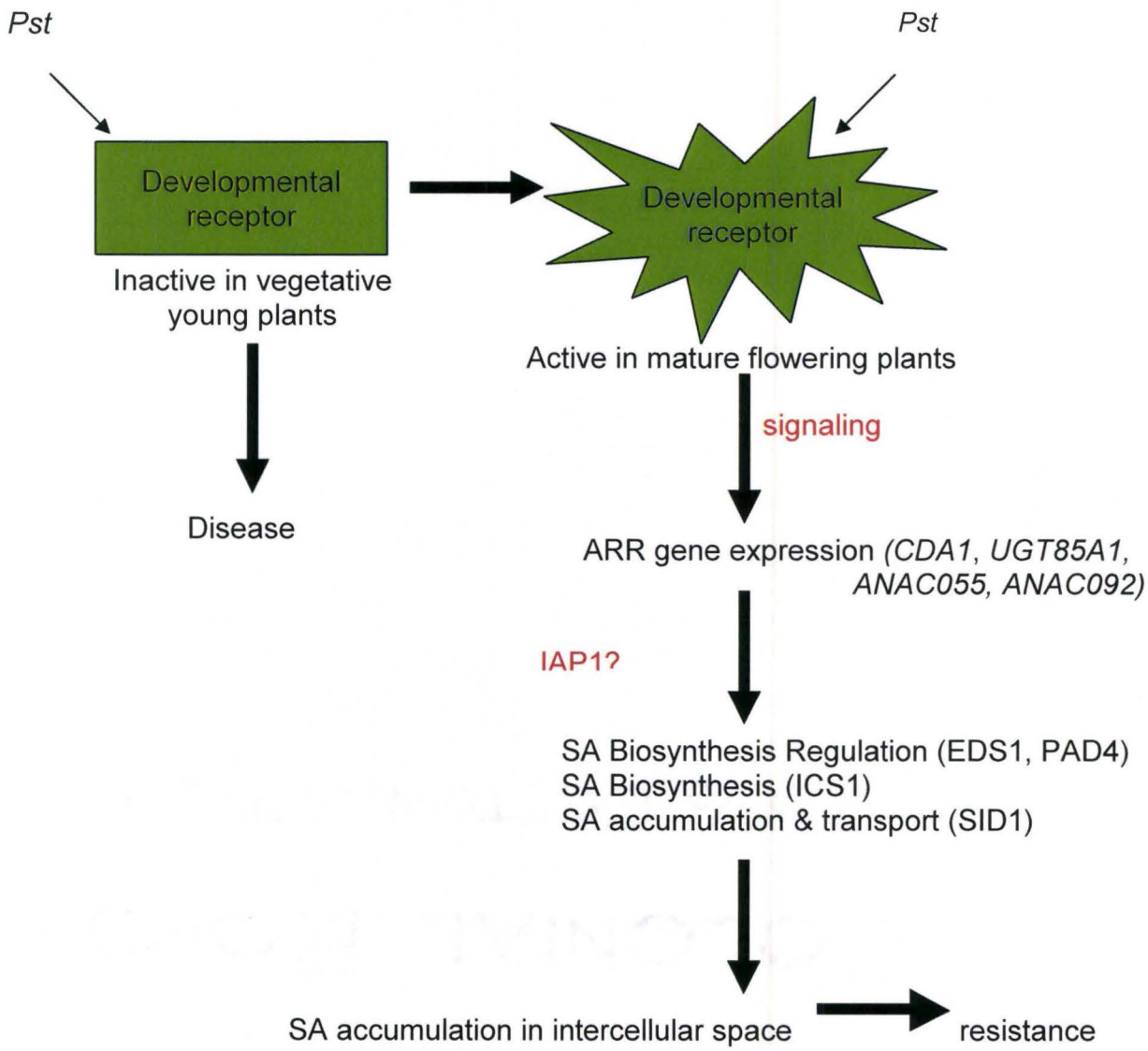
basal resistance and R gene-mediated resistance at 48 hpi suggesting that *IAP1* is more likely involved in the regulation of SA than the biosynthesis. The *iap1-1* mutation also reveals differences between the three pathways as it causes an almost complete loss of ARR, a partial loss of R gene-mediated resistance, but does not affect basal resistance to *Pst* as increased susceptibility was not observed. This evidence suggests that *IAP1*-dependent SA accumulation is important for a robust ARR and R gene-mediated resistance response but is not required for basal resistance. It makes evolutionary sense that these pathways are related as it would be easier to build on existing pathways versus building a completely new pathway. The discovery that intercellular SA is required for R gene-mediated resistance is novel as intercellular SA accumulation has only previously been associated with ARR. The fact that intercellular SA accumulation was observed during R gene-mediated resistance and not basal resistance (even at 48 hpi) is also interesting as it is believed that the defense mechanisms employed by these two pathways are very similar (Ham et al., 2007; Tsuda et al., 2008). These discoveries emphasize that there is still much unknown about R gene-mediated resistance.

While studying SA accumulation in *iap1-1* mutants the Cameron lab became one of the first labs to begin using a novel and more efficient method for measuring SA which involves a biosensor. Publication of these data will provide additional evidence that this SA analysis technique does work and will make SA analysis available to many labs as this method requires no expensive equipment.

This thesis also optimized a new protocol for the Cameron lab for measuring *in planta* bacterial levels that replaces the previously labour-intensive grinding of leaf disc method with a diffusion method that uses a detergent to release bacteria from leaf discs. This method makes it possible to measure bacterial density in twice as many individuals in the same amount of time compared to the previous method. Lastly, a mutant screen was designed that was capable of producing preliminary results two days sooner than previous screens.

IAP1 was hypothesized to be a positive regulator in the ARR pathway and therefore may regulate expression of genes required for ARR. RT-PCR expression data indicated that expression of *ICS1, SID1, EDS1, ANAC055*, *ANAC092*, *CDA1*, and *UGT85A1* was similar in *iap1-1* compared to wild type suggesting that *IAP1* may be downstream of these genes. *ICS1, SID1,* and *EDS1* are all required for SA accumulation. *UGT85A1* is hypothesized to be involved in the conjugation of glucose to proteins and secondary metabolites, including SA. *CDA* genes play a role in RNA editing by converting cytidine to uridine. *ANAC055* and *ANAC092* are believed to be involved in development.

Figure 27. ARR model based on SA rescue experiments (Cameron and Zaton, 2004), the ARR microarray, and expression of ARR genes in *iap1-1* (Carviel et al., 2009).



The identity of *IAP1* is currently unknown, however mapping is ongoing. There are several possible reasons why map based cloning of this gene has been so challenging. Biotic stress such as pathogen infection has been shown to induce genome instability in diverse species such as *Arabidopsis*, tobacco, and maize (Lebel et al., 1993; Lucht et al., 2002; Puchta et al., 1995; Ries et al., 2000; Kovalchuk et al., 1998). *Hyaloperonospora arabidopsidis* has been shown to increase the frequency of somatic recombination in *Arabidopsis* (Lucht et al., 2002) while both tobacco mosaic virus and oilseed rape mosaic virus have been shown to increase homologous recombination in tobacco by three fold (Kovalchuk et al., 2003). Genetic changes created as a result of pathogen-induced genome instability have also been shown to be inherited in the subsequent generations (Kovalchuk et al., 2003). It has been hypothesized that pathogen-induced genetic instability may lead to adaptive mutations and thus be part of a plants resistance response in some cases (Dong, 2004). Furthermore, these mutations are thought to be targeted to genes involved in disease resistance such as R genes (Boyko et al., 2007). As the screen for ARR mutants included pathogen inoculation, it is possible that some of the seeds may have inherited different pathogen-induced genetic changes near or in genes involved in disease resistance such as *IAP1*. These changes could affect map based cloning results if *IAP1* is located in different positions in some individuals in the mapping population. It is unknown whether *Pst* can induce genetic instability in *Arabidopsis,* but it is a possibility, although other resistance genes have been

map-based cloned without reporting this issue (Krattinger et al., 2009). The second problem with map-based cloning to identify *IAP1* is that stressed wild type plants can sometimes appear to be ARR-defective. Map-based cloning is very sensitive so even one incorrectly genotyped individual can produce misleading results. Complementation experiments suggest that *IAP1* is not *EDS1, PAD4, SID1*, or *SID2.* Due to some of the problems with the assay mentioned previously however they cannot be ruled out completely at this point in time and it is possible that *IAP1* could be a different allele of *EDS1, PAD4, SID1,* or *SID2.*

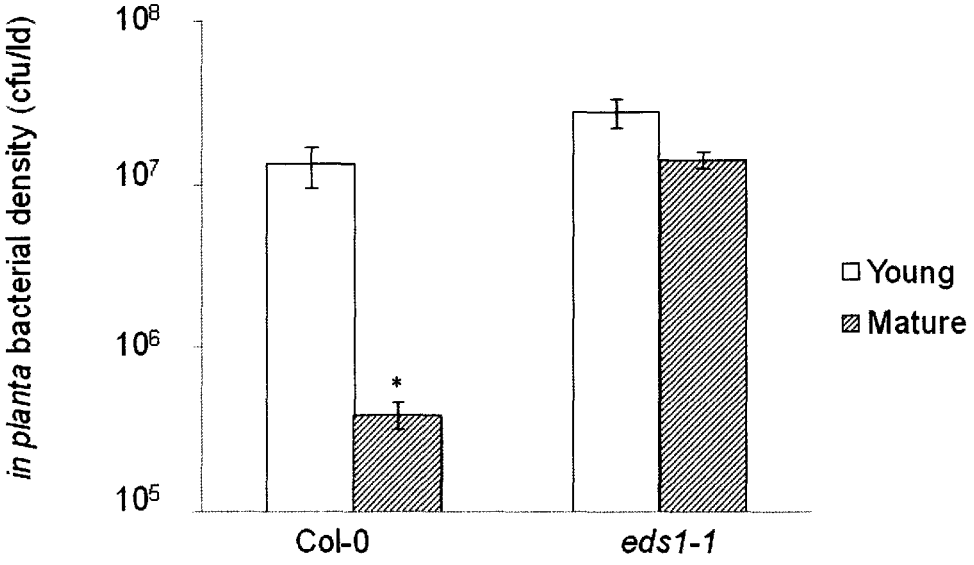
IAP1 is believed to be located between 17,938,268bp and 18,133,423bp on chromosome four. Fine mapping has been challenging due to limitations in the ARR screen, therefore several alternate methods are being used in tandem to identify *IAP1,* including transiently expressing candidate genes in *iap1-1* and assaying for ARR. If *IAP1* is one of the candidate genes the ARR pathway should be rescued in *iap1-1* when it is transiently expressed. Successful mapping of *IAP1* will provide insight into the role of *IAP1* during the ARR response.

In the near future I recommend that time and effort be put into identifying *IAP1.* The transient gene expression assay described above would be used to identify *IAP1.* Two methods of identifying *IAP1* are required to confirm that the correct gene was identified. After *IAP1* is identified by the transient gene expression assay it would be sequenced in *iap1*-1to confirm the mutation.

Overall, *IAP1* is a potential candidate for genetic manipulation to create disease resistant crop plants (possibly by inducing ARR in young plants) that will require fewer pesticide applications. This research has greatly enhanced our knowledge of ARR and has even provided new insights into the previously well-studied R gene-mediated resistance response. It is hoped that this knowledge will be used in the creation of pathogen-resistant crop plants.

Appendix A: Replicate Experiments

Figure 7 Replicate Experiments



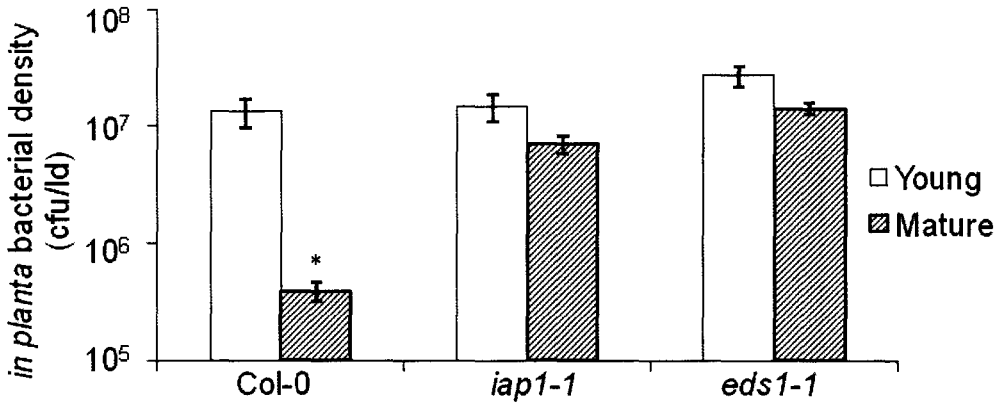
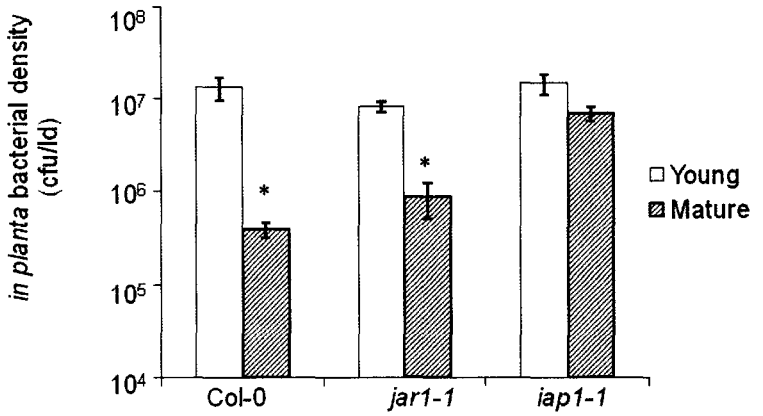
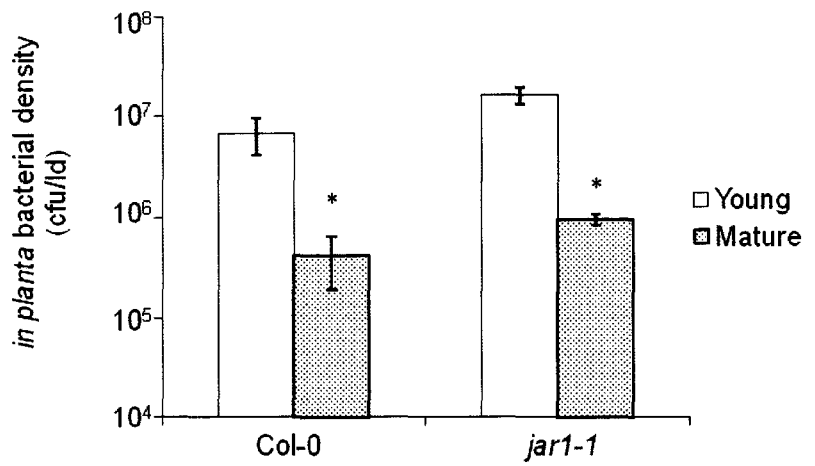


Figure 8 Replicate Experiments





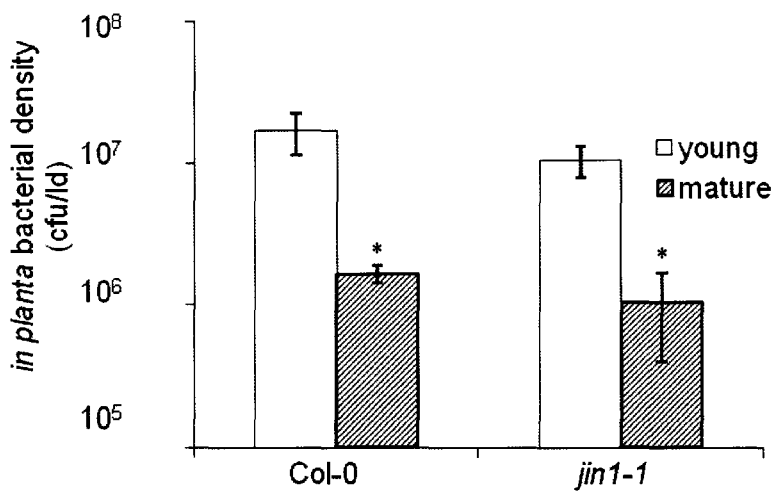
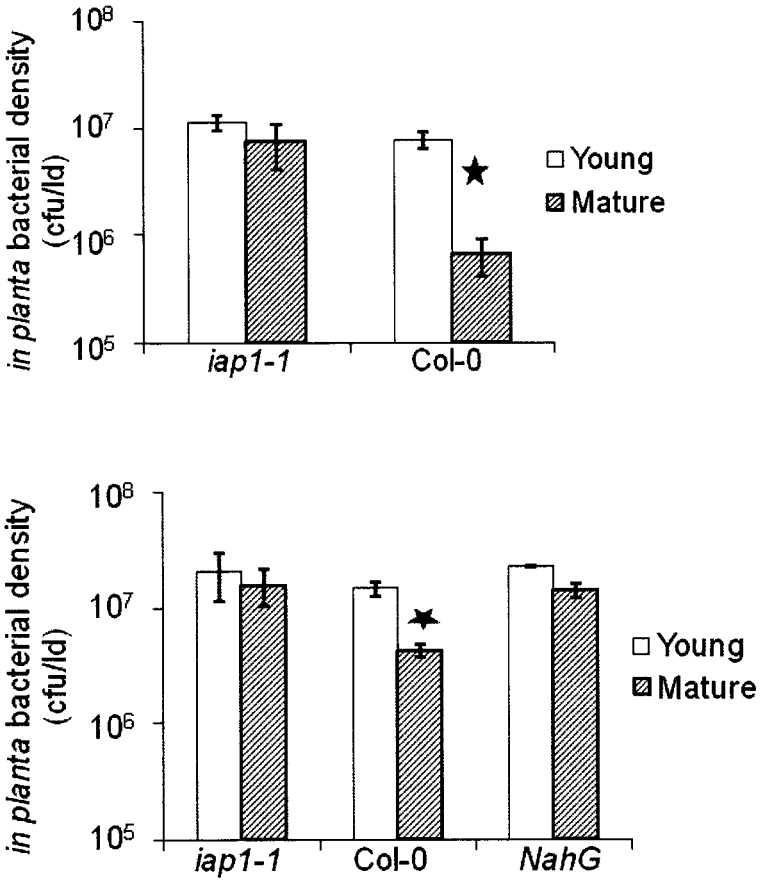
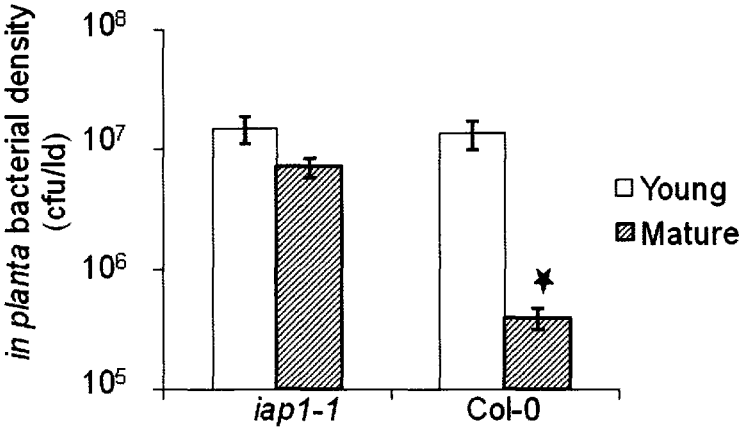


Figure 11 Replicate Experiments




in planta bacterial density (cfu/ld)
10^8
10^7
10^6
10^5
iap1-1
Col-0
Young
Mature

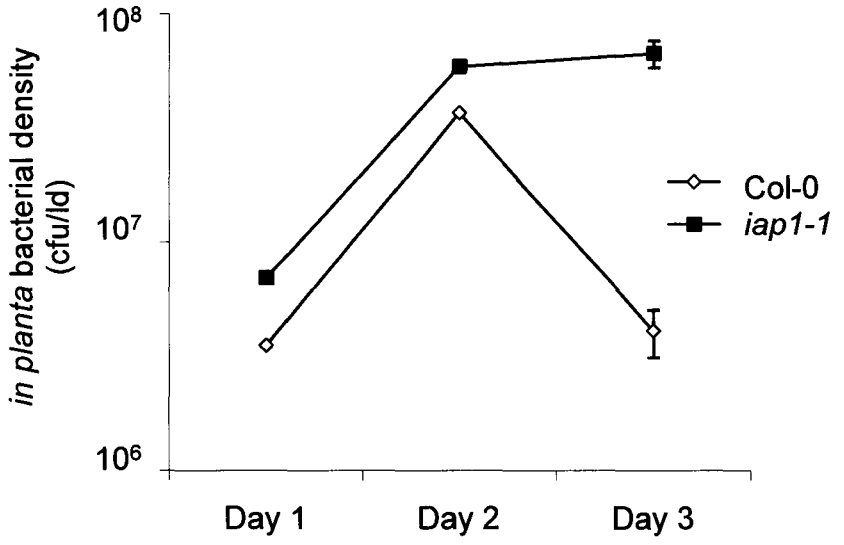
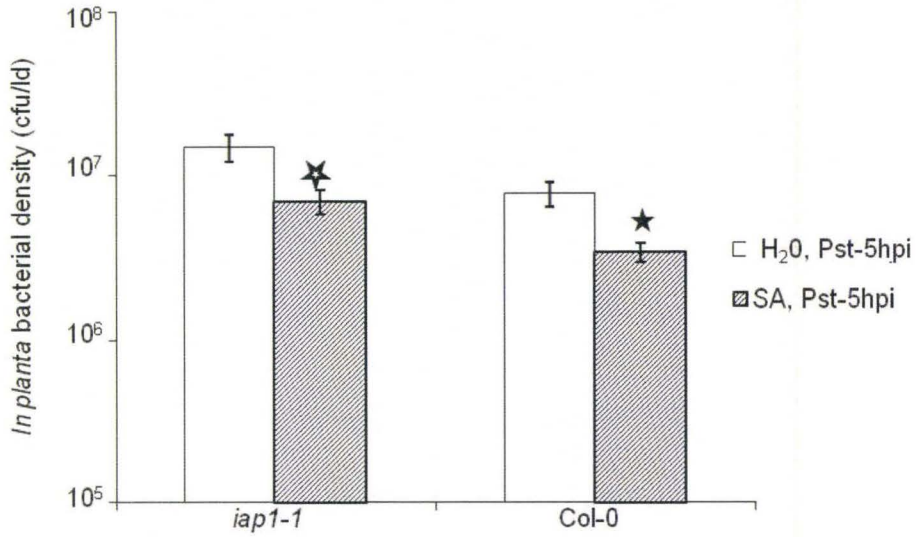

in planta bacterial density (cfu/ld)
10^8
10^7
10^6
Day 1
Day 2
Day 3
Col-0
iap1-1

Figure 12 Replicate Experiments



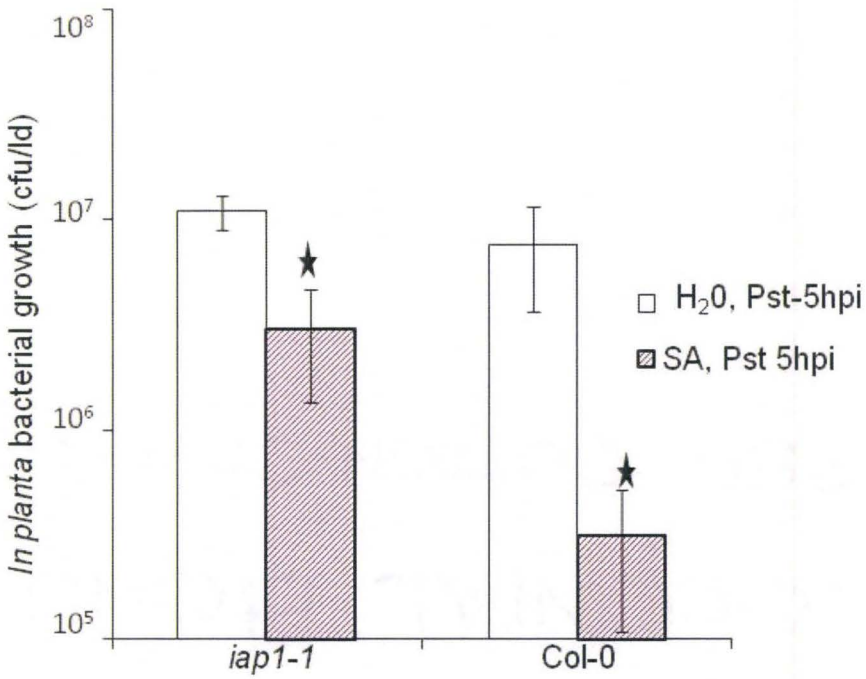
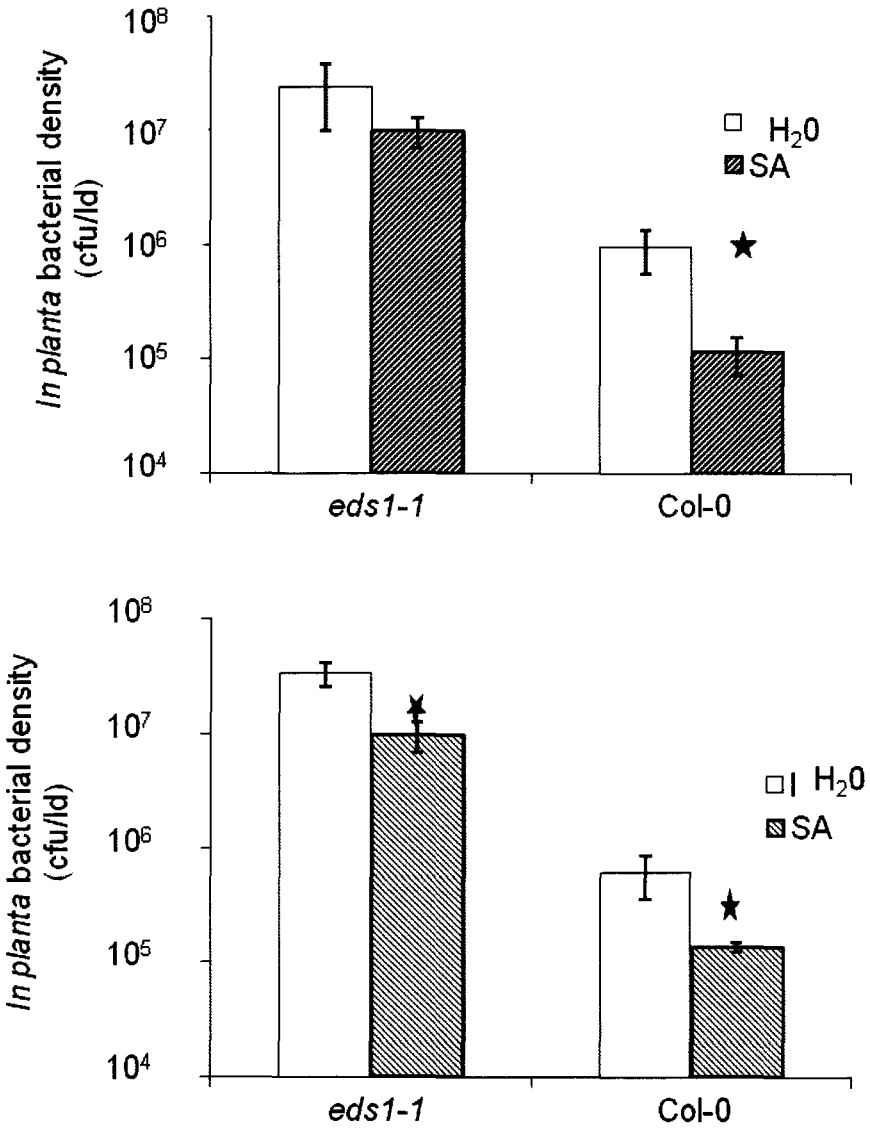


Figure 13 Replicate Experiments



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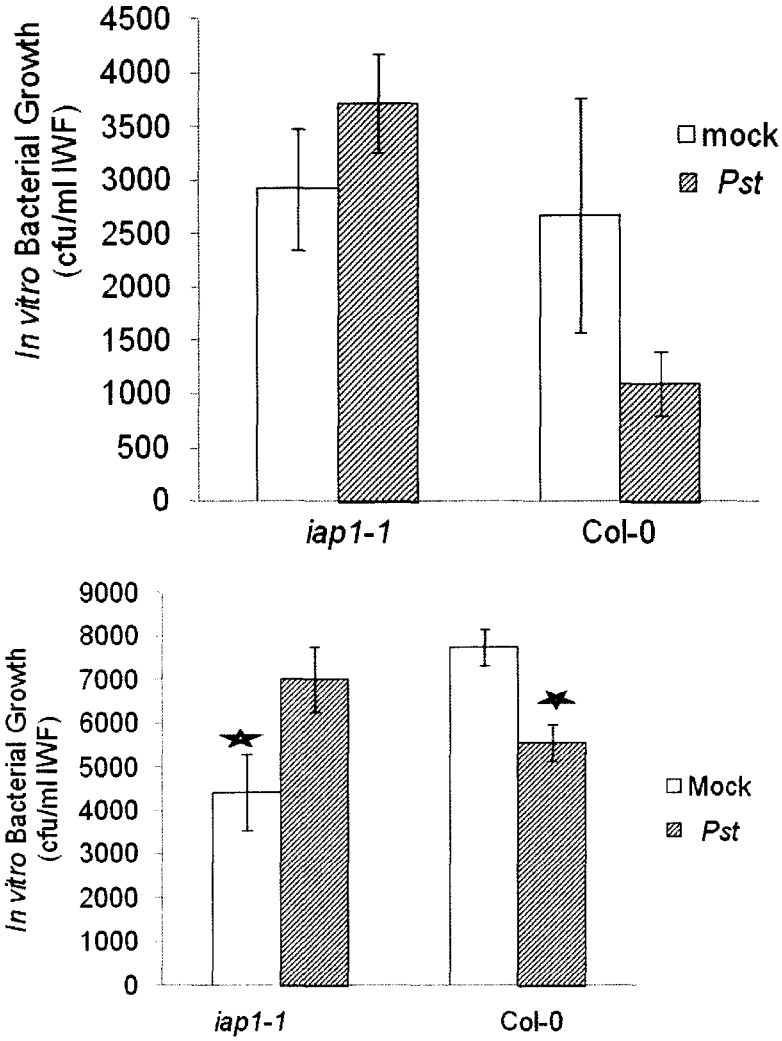
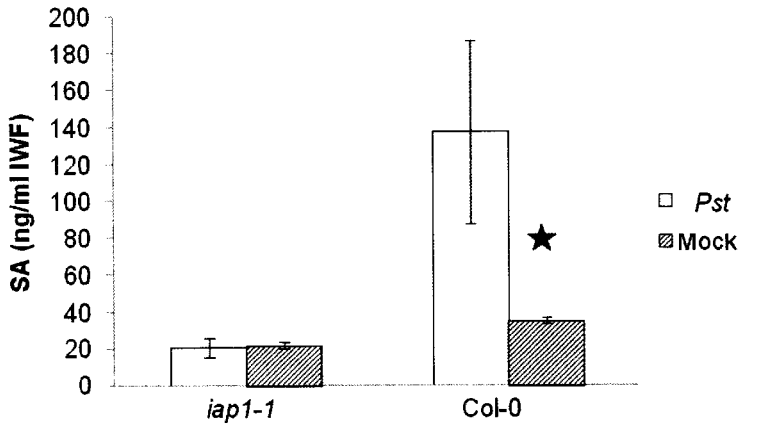
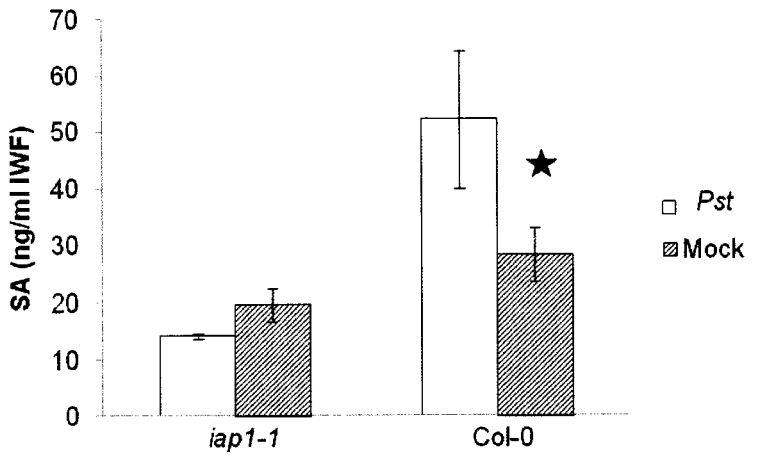


Figure 15 Replicate Experiments





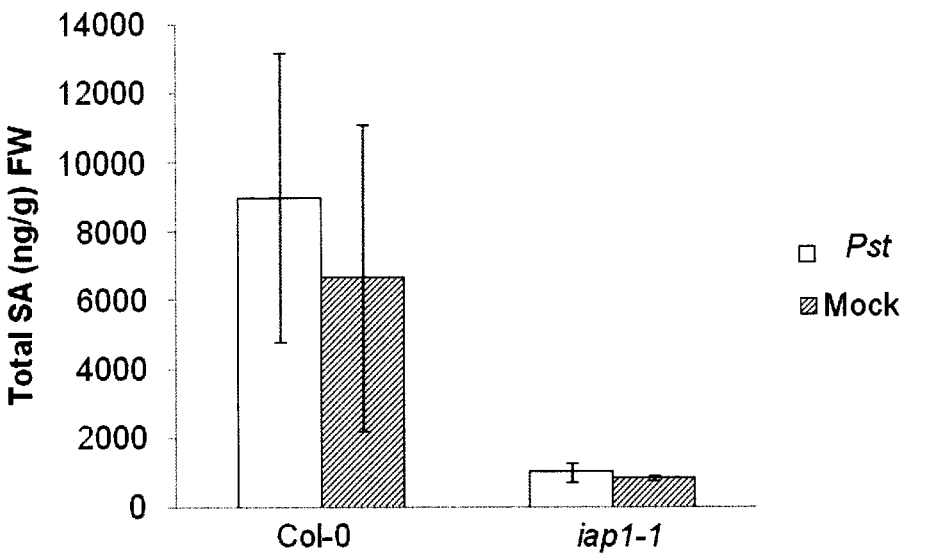
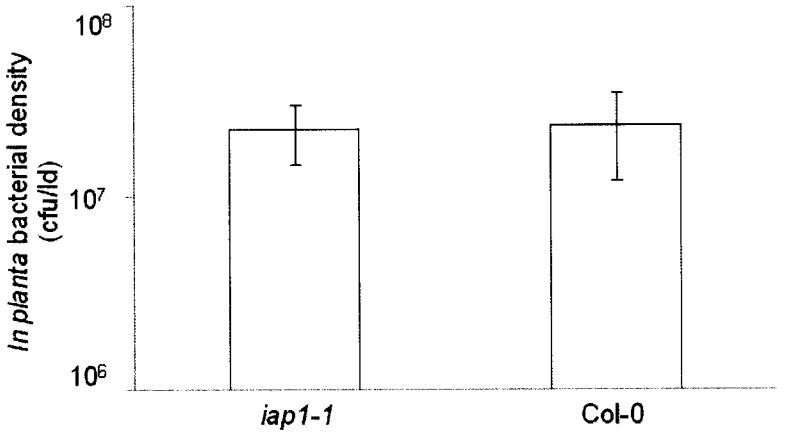
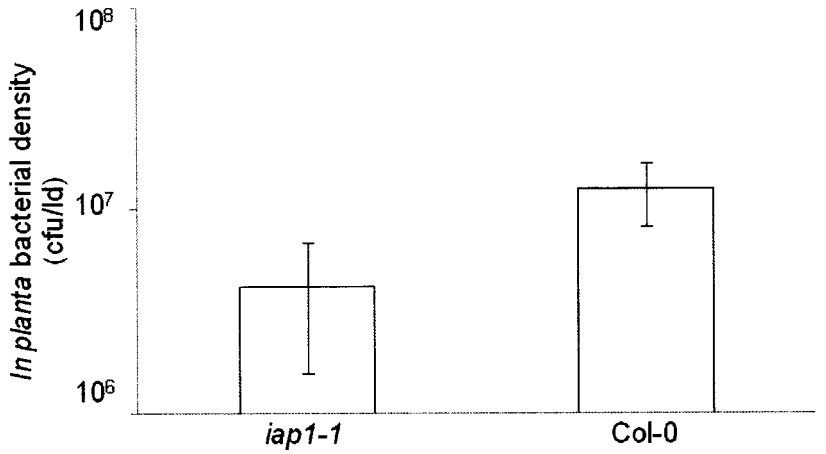


Figure 16 Replicate Experiments





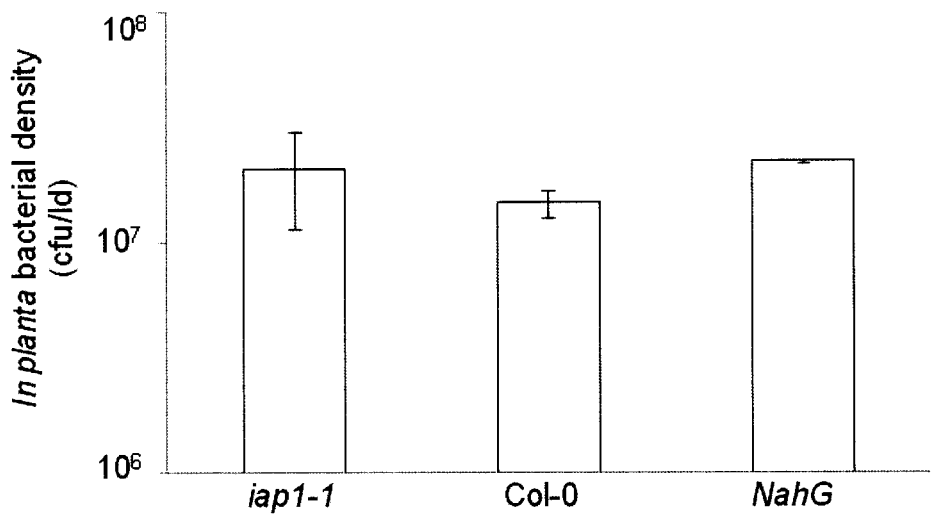


Figure 17 Replicate Experiments

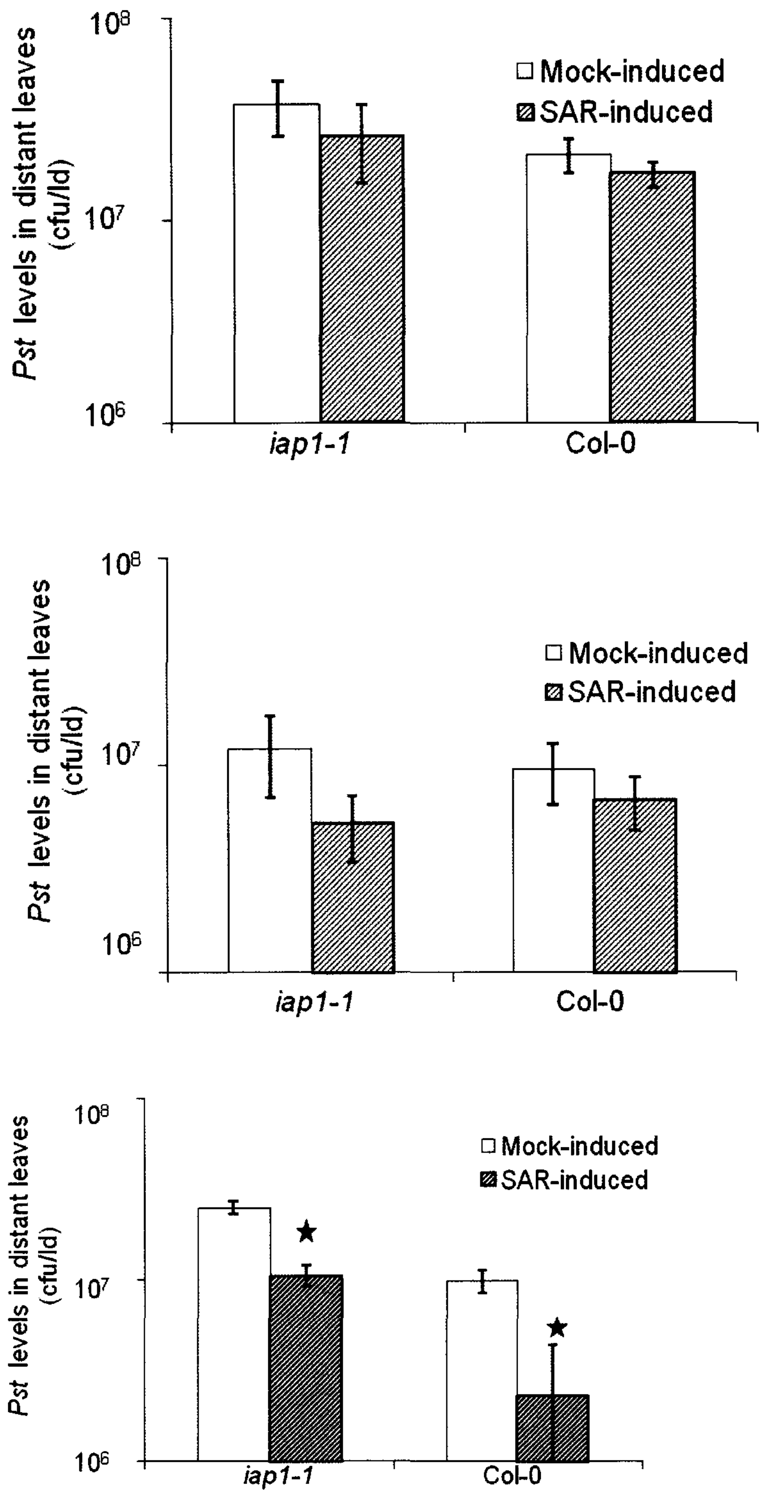
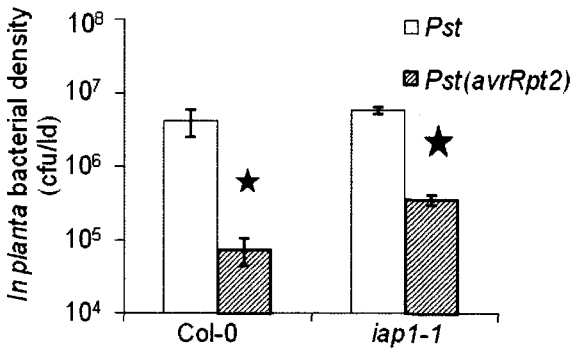
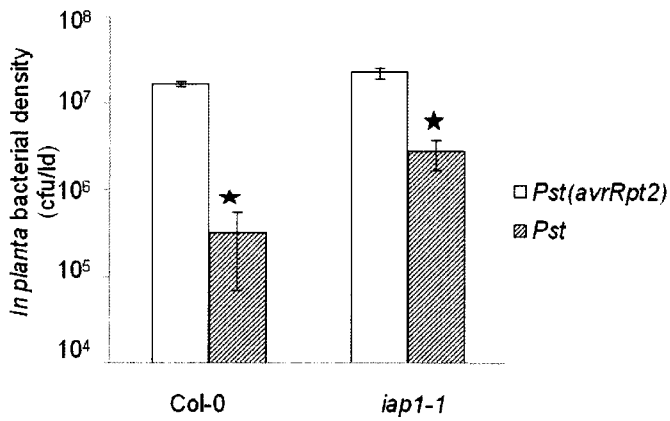
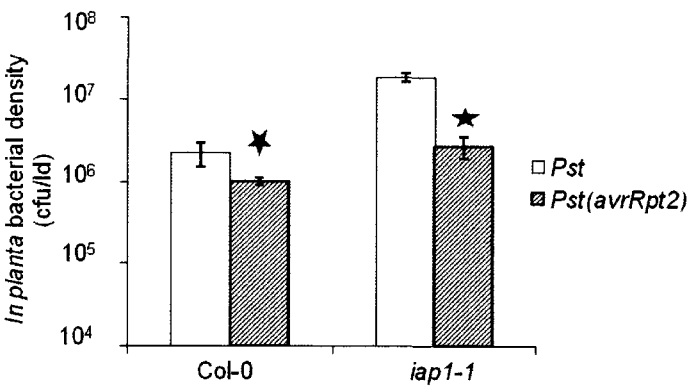
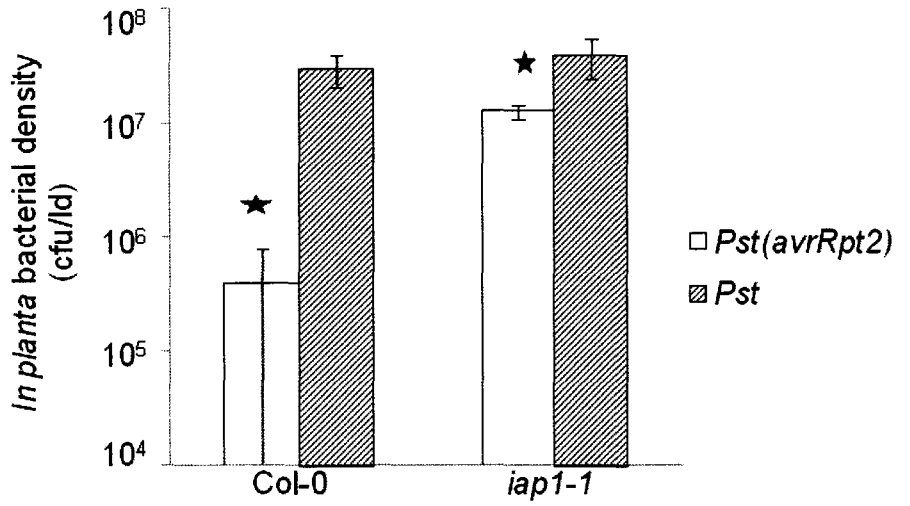


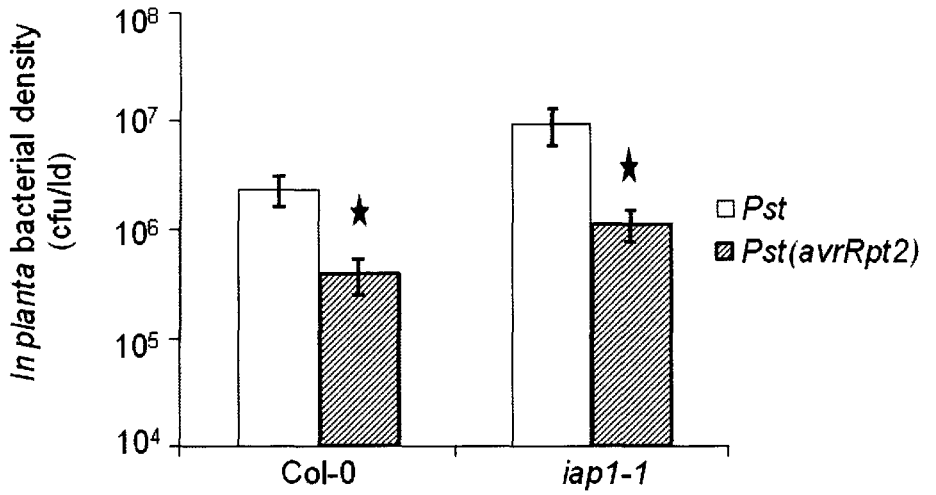
Figure 18 Replicate Experiments

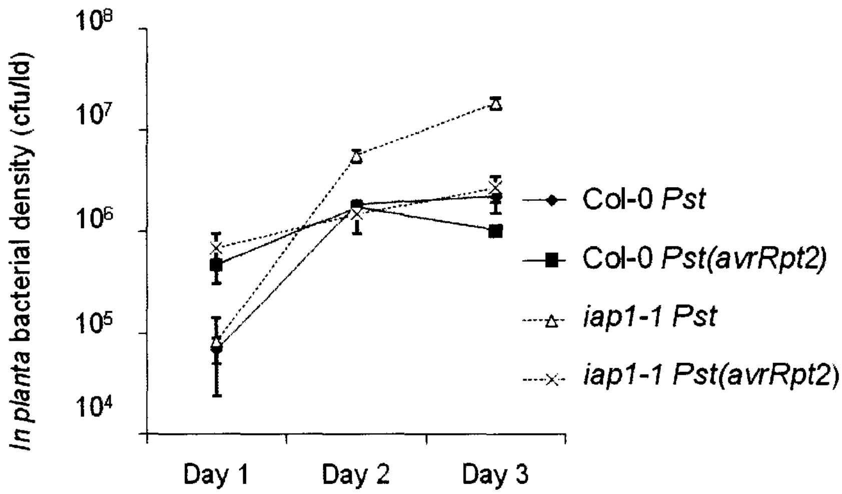


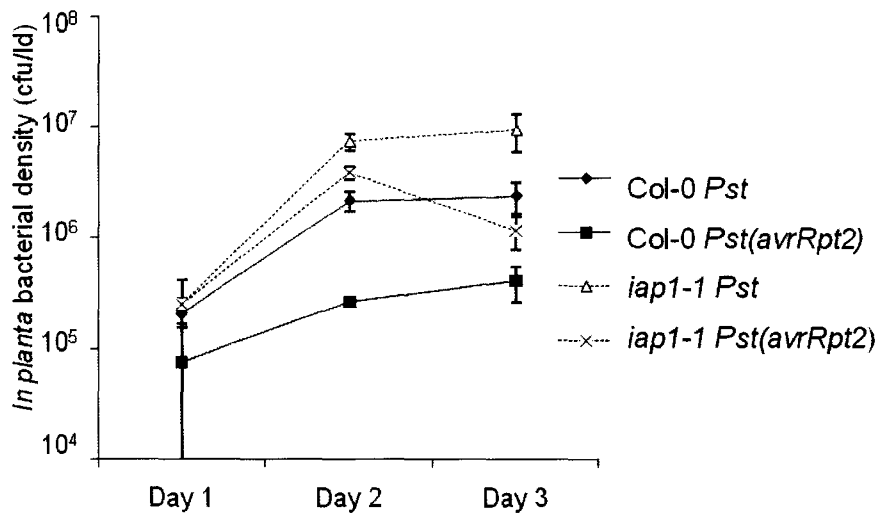





In planta bacterial density (cfu/ld)
10^8
10^7
10^6
10^5
10^4
Col-0
iap1-1
Pst(avrRpt2)
Pst


In planta bacterial density (cfu/ld)
10^8
10^7
10^6
10^5
10^4
Col-0
iap1-1
Pst
Pst(avrRpt2)


In planta bacterial density (cfu/ld)
10⁸
10⁷
10⁶
10⁵
10⁴
Day 1
Day 2
Day 3
Col-0 Pst
Col-0 Pst(avrRpt2)
iap1-1 Pst
iap1-1 Pst(avrRpt2)


In planta bacterial density (cfu/ld)
10⁸
10⁷
10⁶
10⁵
10⁴
Day 1
Day 2
Day 3
Col-0 Pst
Col-0 Pst(avrRpt2)
iap1-1 Pst
iap1-1 Pst(avrRpt2)

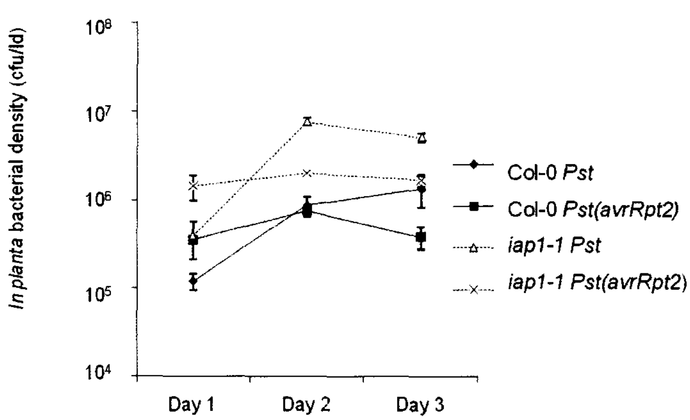

In planta bacterial density (cfu/ld)
10⁸
10⁷
10⁶
10⁵
10⁴
Day 1
Day 2
Day 3
Col-0 Pst
Col-0 Pst(avrRpt2)
iap1-1 Pst
iap1-1 Pst(avrRpt2)

Figure 19 Replicate Experiments

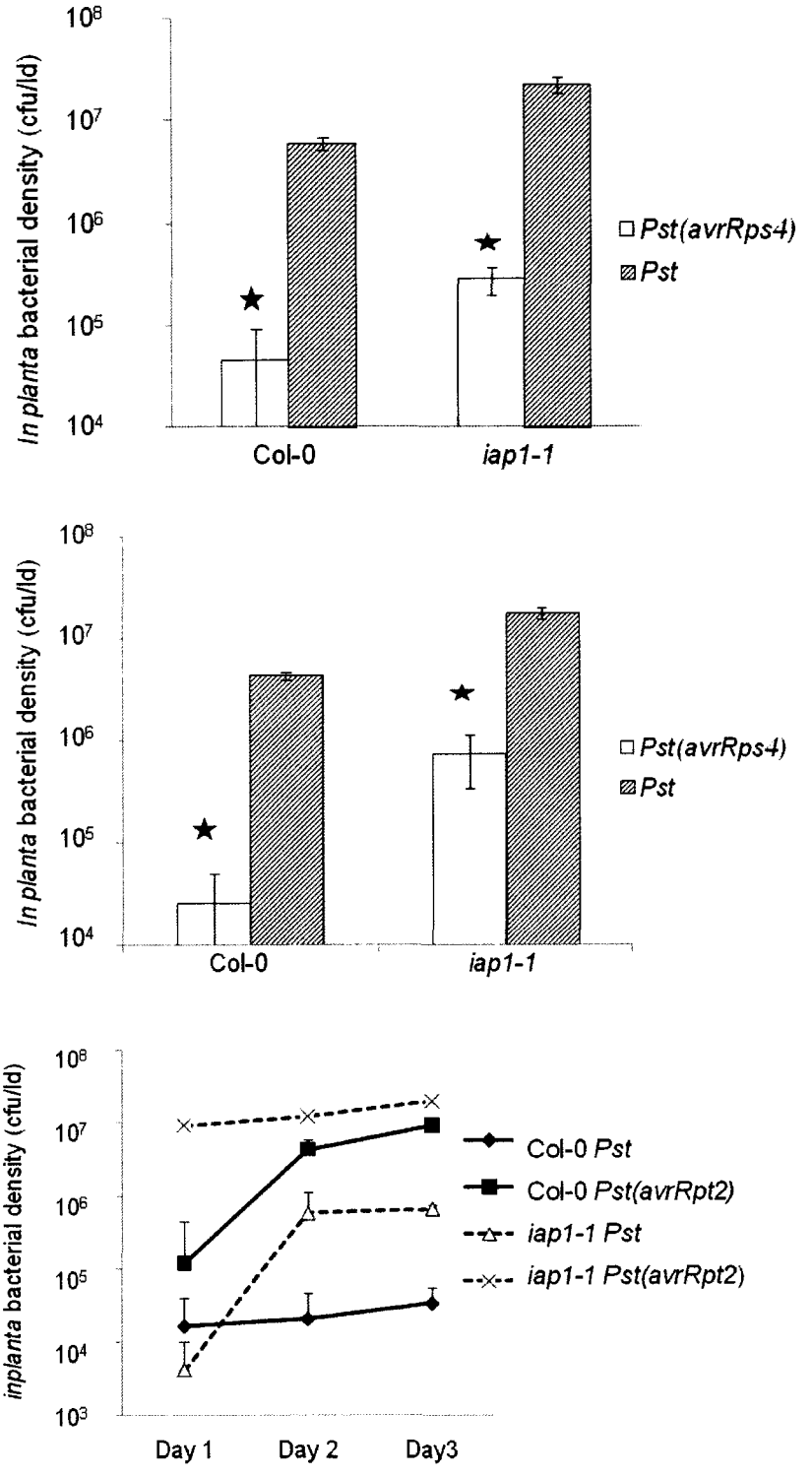
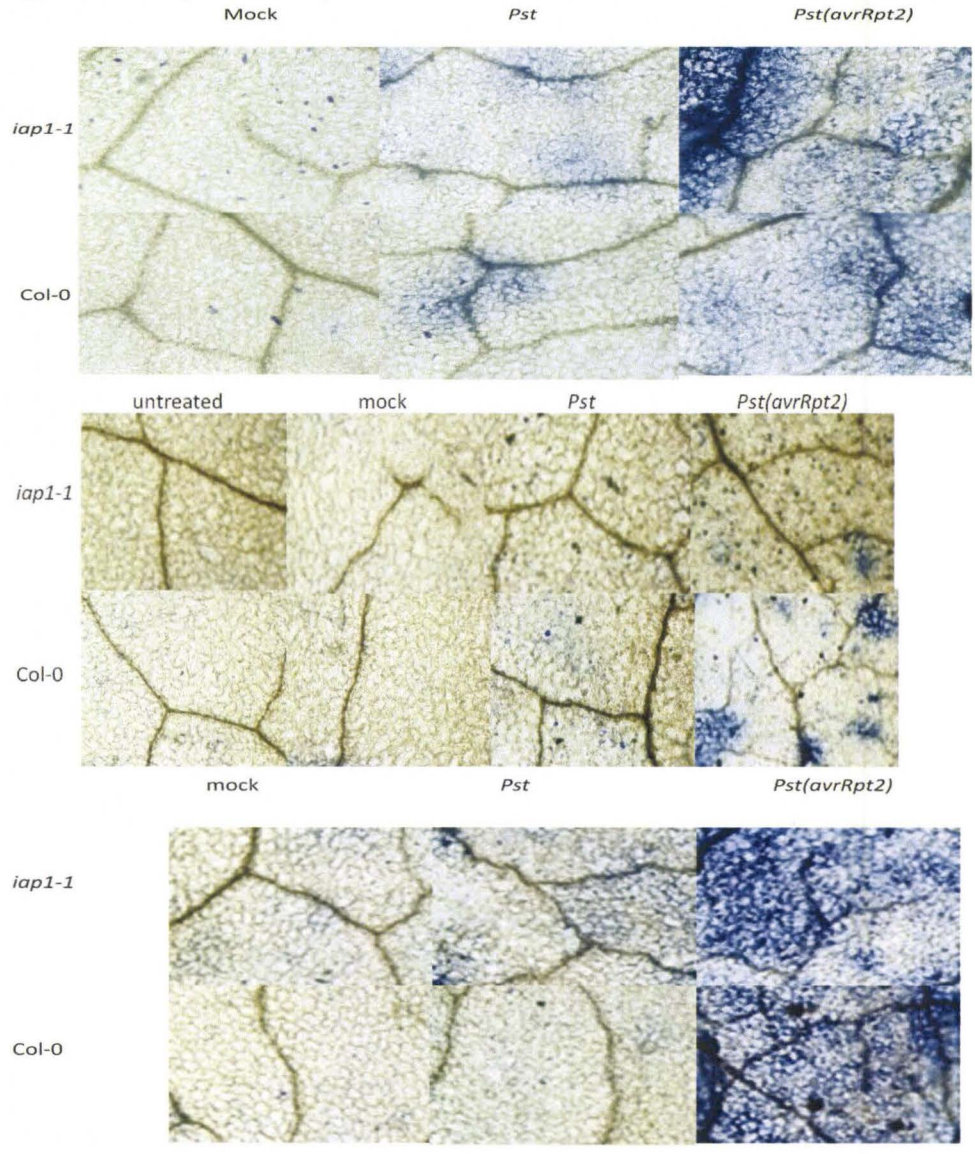
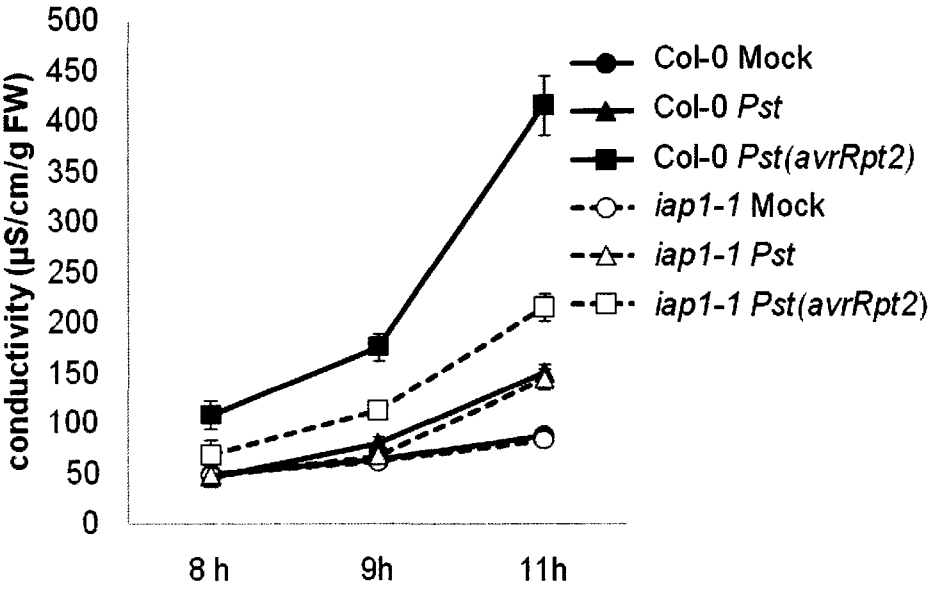


Figure 20 Replicate Experiments




conductivity (µS/cm/g FW)
500
450
400
350
300
250
200
150
100
50
0
8 h
9h
11h
Col-0 Mock
Col-0 Pst
Col-0 Pst(avrRpt2)
iap1-1 Mock
iap1-1 Pst
iap1-1 Pst(avrRpt2)

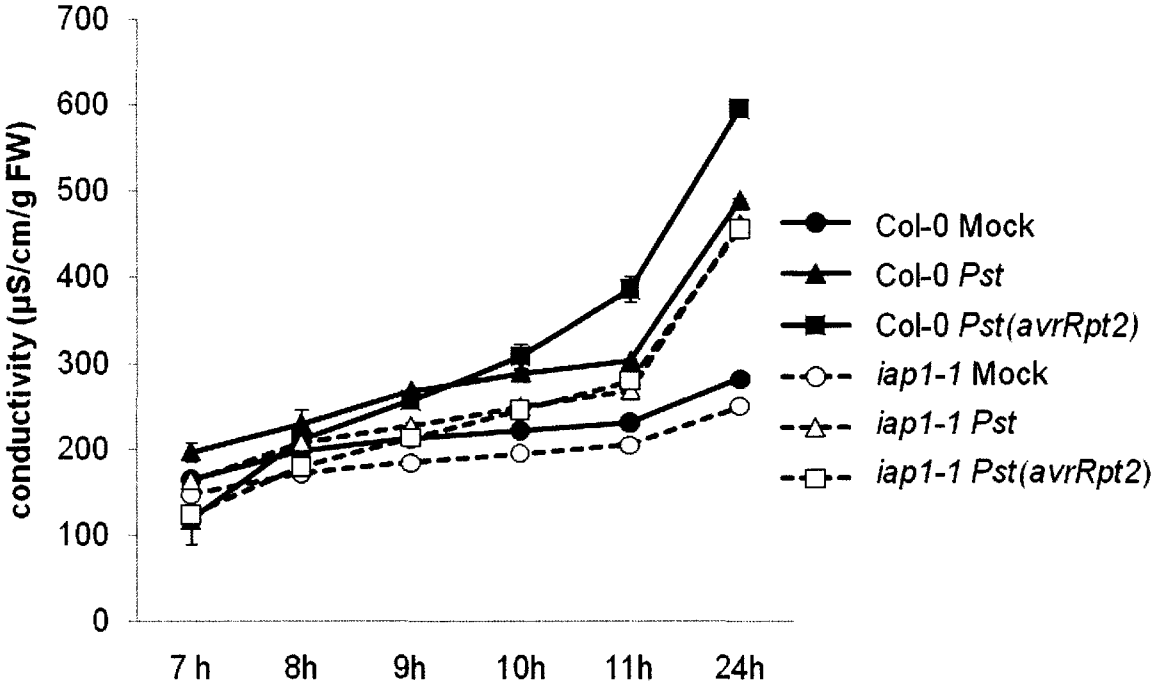

conductivity (µS/cm/g FW)
700
600
500
400
300
200
100
0
7 h
8h
9h
10h
11h
24h
Col-0 Mock
Col-0 Pst
Col-0 Pst(avrRpt2)
iap1-1 Mock
iap1-1 Pst
iap1-1 Pst(avrRpt2)

Figure 21 Replicate Experiments

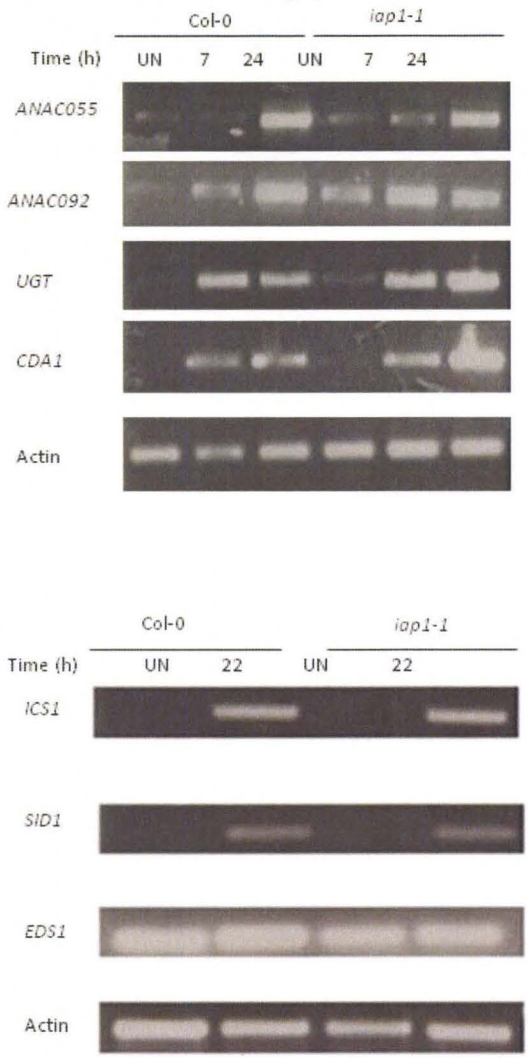
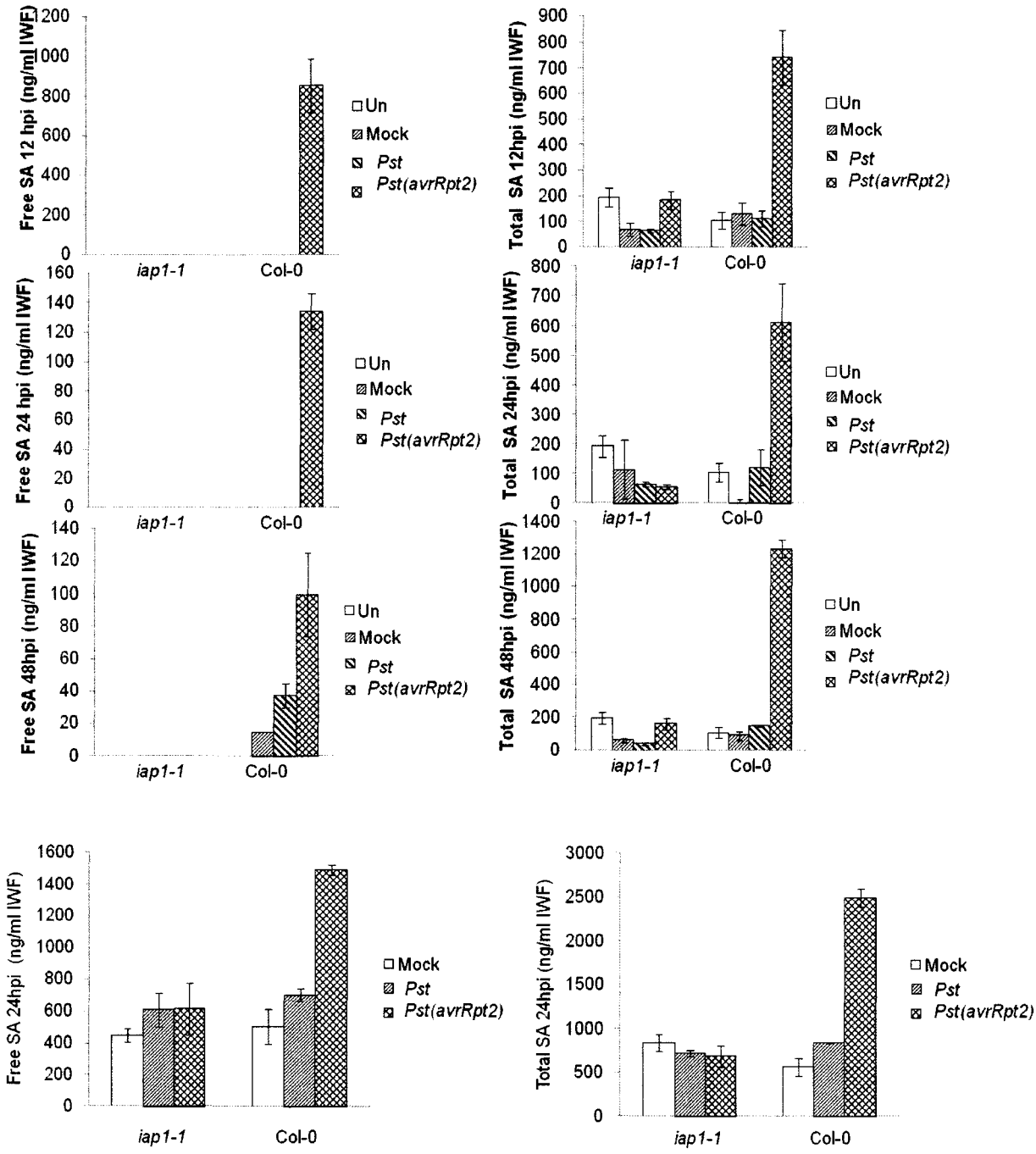
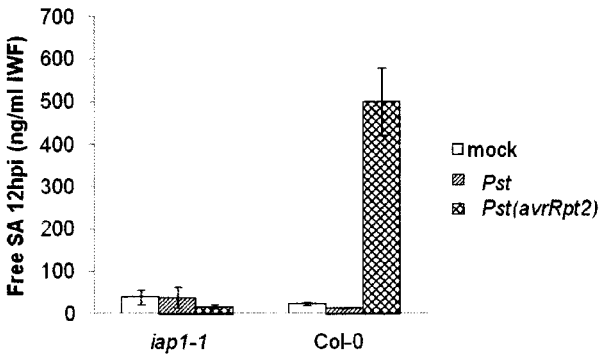
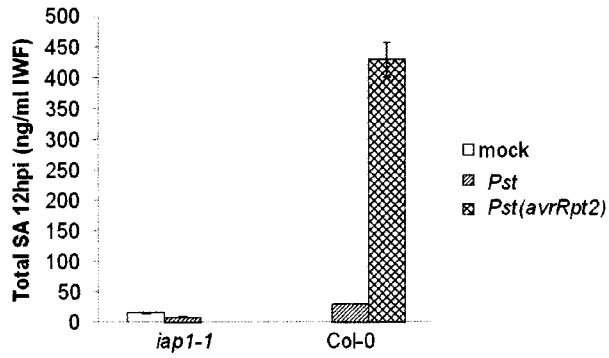
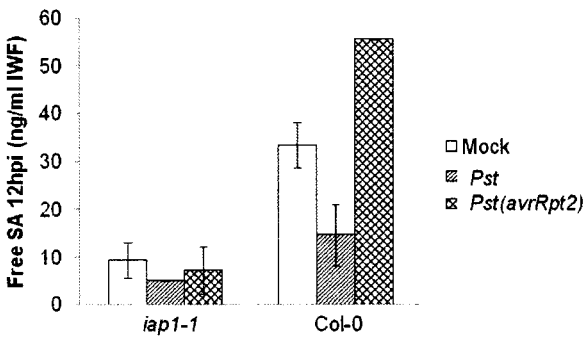


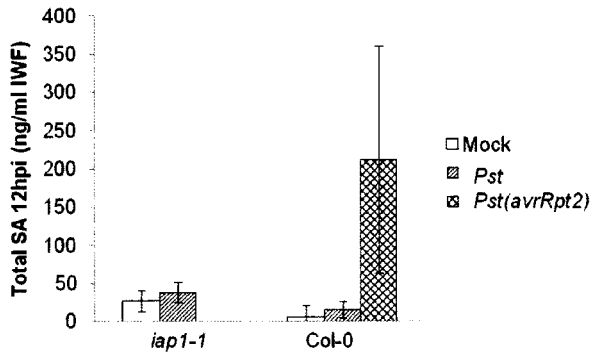
Figure 22 Replicate Experiments

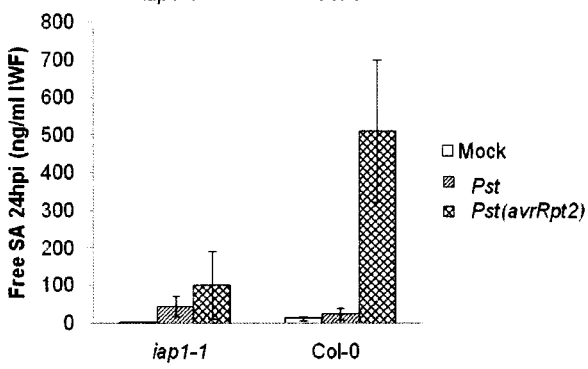



Free SA 12hpi (ng/ml IWF)
700
600
500
400
300
200
100
0
iap1-1
Col-0
mock
Pst
Pst(avrRpt2)


Total SA 12hpi (ng/ml IWF)
500
450
400
350
300
250
200
150
100
50
0
iap1-1
Col-0
mock
Pst
Pst(avrRpt2)


Free SA 12hpi (ng/ml IWF)
60
50
40
30
20
10
0
iap1-1
Col-0
Mock
Pst
Pst(avrRpt2)


Total SA 12hpi (ng/ml IWF)
400
350
300
250
200
150
100
50
0
iap1-1
Col-0
Mock
Pst
Pst(avrRpt2)


Free SA 24hpi (ng/ml IWF)
800
700
600
500
400
300
200
100
0
iap1-1
Col-0
Mock
Pst
Pst(avrRpt2)

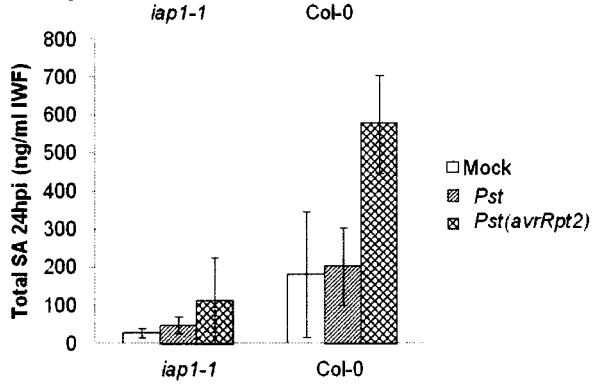
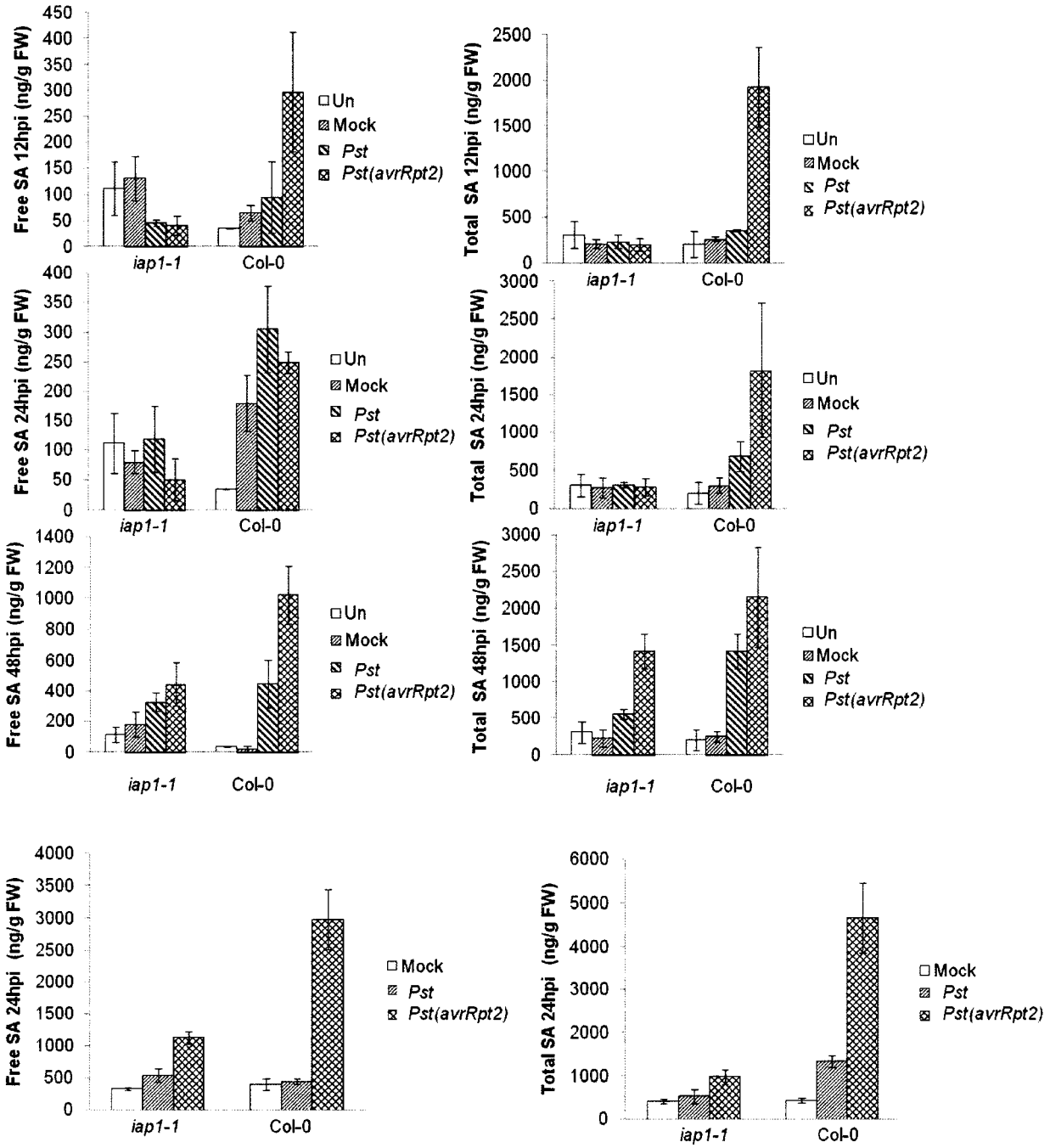
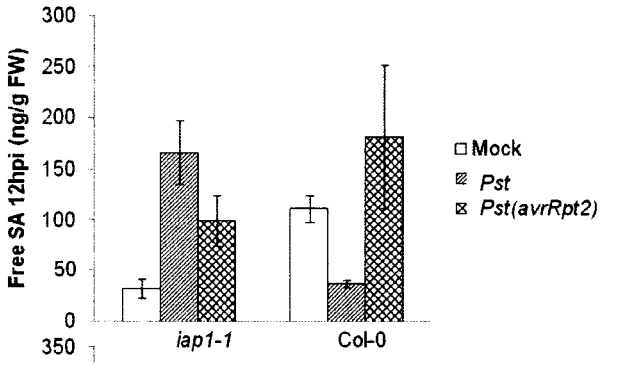
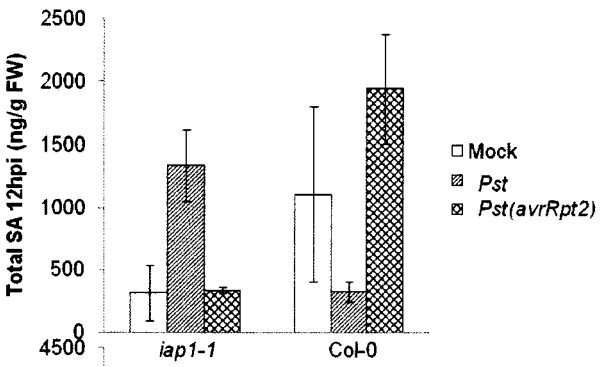
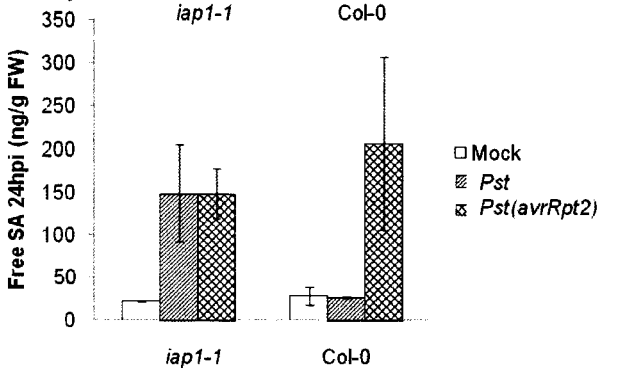

Total SA 24hpi (ng/ml IWF)
800
700
600
500
400
300
200
100
0
iap1-1
Col-0
Mock
Pst
Pst(avrRpt2)

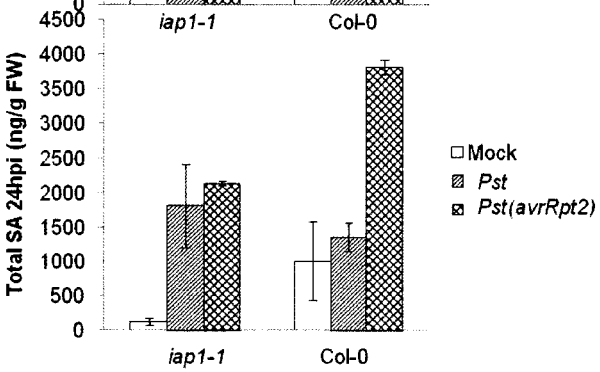
Figure 23 Replicate Experiments

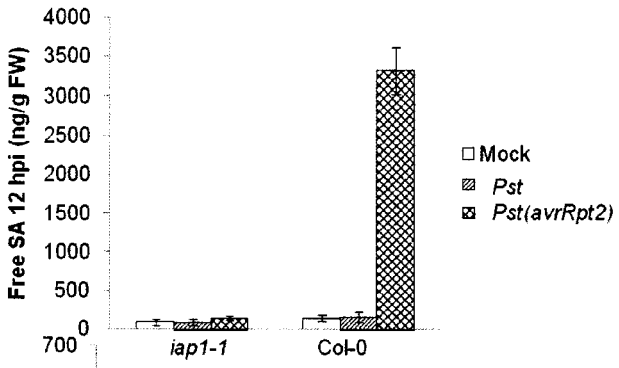


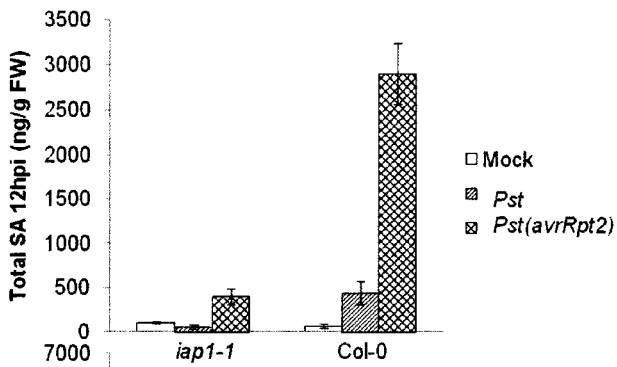

Free SA 12hpi (ng/g FW)
300
250
200
150
100
50
0
iap1-1
Col-0
Mock
Pst
Pst(avrRpt2)

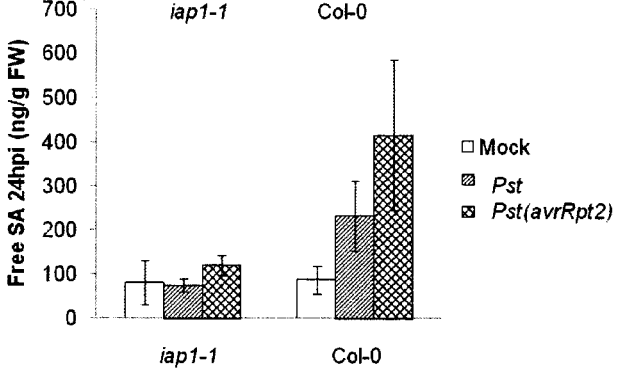

Total SA 12hpi (ng/g FW)
2500
2000
1500
1000
500
0
iap1-1
Col-0
Mock
Pst
Pst(avrRpt2)


Free SA 24hpi (ng/g FW)
350
300
250
200
150
100
50
0
iap1-1
Col-0
Mock
Pst
Pst(avrRpt2)


Total SA 24hpi (ng/g FW)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
iap1-1
Col-0
Mock
Pst
Pst(avrRpt2)


Free SA 12 hpi (ng/g FW)
4000
3500
3000
2500
2000
1500
1000
500
0
iap1-1
Col-0
Mock
Pst
Pst(avrRpt2)


Total SA 12hpi (ng/g FW)
3500
3000
2500
2000
1500
1000
500
0
iap1-1
Col-0
Mock
Pst
Pst(avrRpt2)


Free SA 24hpi (ng/g FW)
700
600
500
400
300
200
100
0
iap1-1
Col-0
Mock
Pst
Pst(avrRpt2)

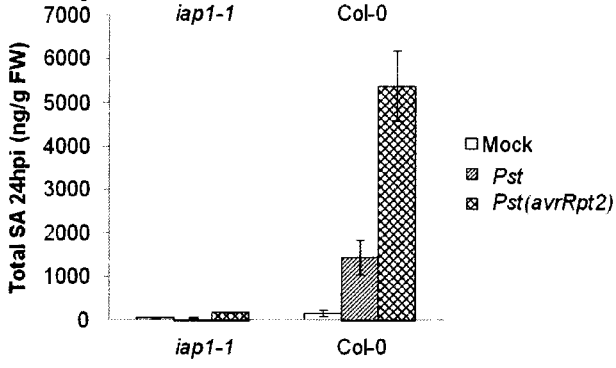
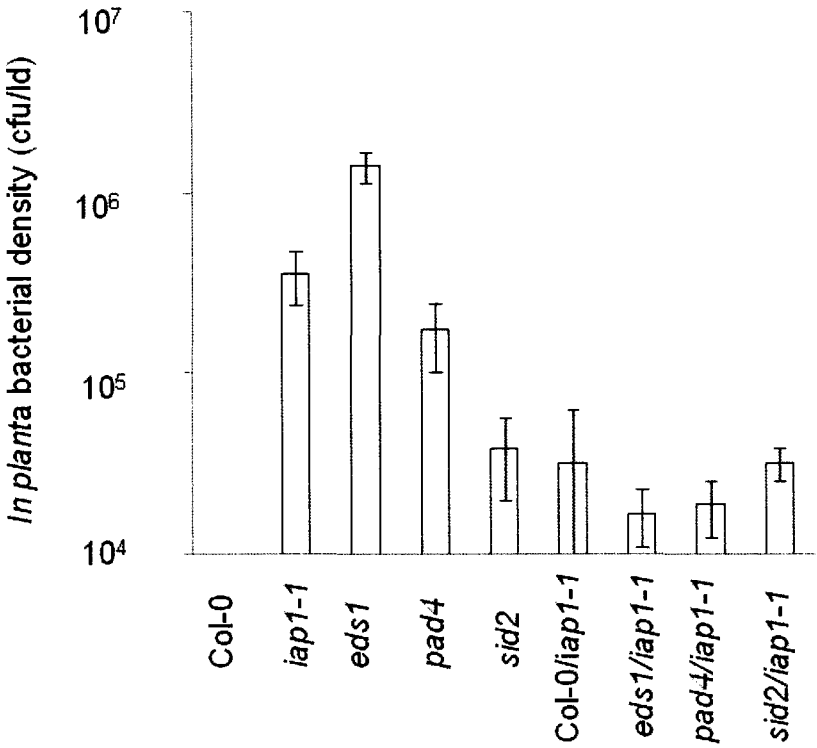
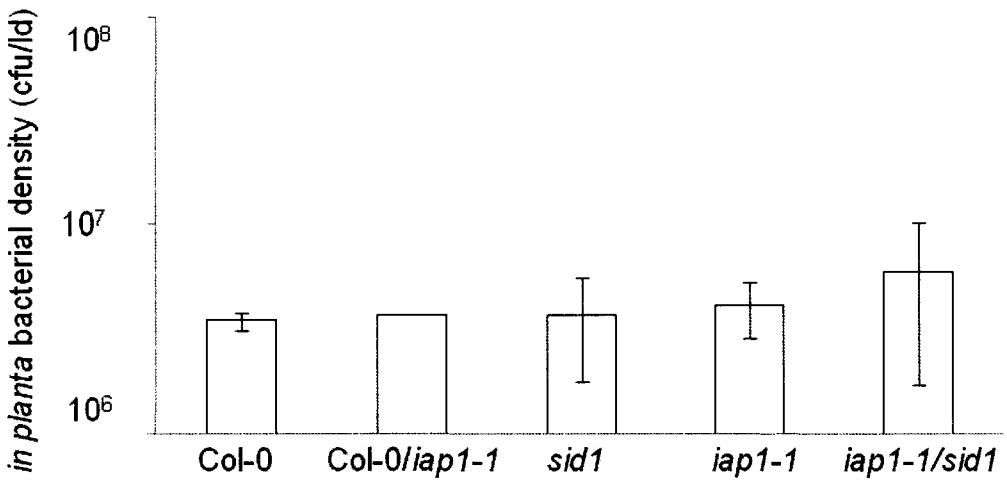

Total SA 24hpi (ng/g FW)
7000
6000
5000
4000
3000
2000
1000
0
iap1-1
Col-0
Mock
Pst
Pst(avrRpt2)

Figure 26 Replicate Experiments





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