ANAC055 AND ANAC092 IN AGE-RELATED RESISTANCE IN *ARABIDOPSIS*

THE ROLE OF TWO NAC TRANSCRIPTION FACTORS DURING AGE-RELATED RESISTANCE IN *ARABIDOPSIS*

By

FADI AL-DAOUD, M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctorate of Philosophy

McMaster University

Doctorate of Philosophy (2010) McMaster University

(Biology) Hamilton, Ontario

TITLE: The role of two NAC transcription factors during age-related resistance in *Arabidopsis*

AUTHOR: Fadi Al-Daoud, M.Sc. (McMaster University)

SUPERVISOR: Dr. Robin K. Cameron

NUMBER OF PAGES: i-xxi , 1-392

Abstract

As *Arabidopsis thaliana* ages it becomes more resistant to virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) bacteria. This is known as age-related resistance (ARR). ARR is associated with flowering and intercellular accumulation of salicylic acid (SA). A microarray experiment identified a number of jasmonic acid/ethylene (JA/ET)-associated genes whose expression was up-regulated during ARR. This thesis explores the role of JA/ET signaling during ARR by characterizing the role of two JA/ET-associated No Apical Meristem Cup-shaped Cotyledons (NAC) transcription factors: ANAC055 and ANAC092. Analysis of *nac* single and double mutants suggests that the NACs play non-redundant roles during ARR. The partial ARR defect of *anac092* is followed one week later by an enhanced ARR response, and this is associated with a delay in flowering. Furthermore, mature 35S:*ANAC092* exhibits increased susceptibility to *Pst*. Collectively, this data suggests that ANAC092 is a negative regulator of ARR and it contributes to positive regulation of flowering and the onset of ARR. The late flowering mutant *luminidependens1* also exhibits a partial ARR defect, suggesting that the autonomous flowering pathway contributes to ARR. Gene expression data suggests that ANAC055 and ANAC092 regulate expression of some JA/ET-associated genes during ARR. The JA/ET signaling mutant *ethylene insensitive2* (*ein2*) exhibits a partial ARR defect and reduced expression of *ANAC055* and *ANAC092*, suggesting that EIN2 is a positive regulator of expression of *ANAC055* and *ANAC092* during ARR. Phytohormone analyses reveal that JA accumulates to similar levels in young and mature wild-type plants after inoculation with *Pst*, suggesting that increased expression of some JA/ET-associated

genes in mature compared to young plants after inoculation with *Pst* is not associated with elevated levels of JA. This thesis contributes to our understanding of ARR by identifying some components of the NAC pathway, exploring the relationship between flowering and ARR, and conducting some phytohormone analysis.

Acknowledgements

I would like to thank my supervisor, Dr. Robin K. Cameron, for giving me the opportunity to work on this fascinating project. Her patience and stimulating and inspiring discussions were greatly appreciated. I would like to thank members of the Cameron lab that created a great working environment, including Dr. A. Mohammad, Dr. H. Shearer, Dr. M. Champigny, K. Haines, J. Carviel, J. Faubert, M. Melas, and the many undergraduate students that worked in the Cameron lab. I would also like to thank my friends and family, including my lovely wife, for their much-needed love and support.

Thesis Organization and Format

This thesis is composed of six chapters. Chapter 1 provides relevant background information. Chapters 2 to 5 are written as papers for publication. Parts of Chapter 2 were published in Carviel et al. (2009), and data from Chapters 3 to 5 will soon be submitted for publication. Chapter 6 summarizes the results of this thesis and outlines possible future research. The appendix includes the results of all the experiments that were performed. This thesis was made possible by the efforts of many people. Each chapter includes a preface that outlines the contributions of the different authors.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ΔR_n: logarithmic florescence signal

°C: degrees Celsius

$\mu g\ ml^{-1}$: micrograms per milliliter

$\mu m^{-2}s^{-1}$: microEinsteins per square meter per second

3': three prime

5': five prime

ABA: abscisic acid

ABFs: ABA Response Element Binding Factors

ABI1: ABA Insenstive1

ABRC: Arabidopsis Resource Center

ACC: 1-aminocyclopropane-1-carboxylic acid

ACO: ACC oxidase

ACS: ACC synthase

AGI: Arabidopsis GenBank Identification

AGRIS: Arabidopsis Gene Regulator Information Server (Ohio State University)

ANAC: Arabidopsis No Apical Meristem Cup-shaped Cotyledon

AOS: allene oxide synthase

ARF: Auxin Response Factor

ARR: age-related resistance

AtCisDB: Arabidopsis Cis-Regulatory Database

ATPRM5: Arabidopsis thaliana Protein Arginine Methyltransferase5

Avr: avirulent

AXR: Auxin Resistant

BA: benzoic acid

BABA: β-aminobutyric acid

BAK1: Brassinosteroid-associated Kinase1

BR: brassinosteroid

BTB/POZ: broad-complex tramtrack bric-a-brac poxvirus and zinc finger

BTH: benzothiadiazole-s-methyl ester

bZIP: basic leucine zipper

C-terminus: carboxyl-terminus

CaMV: Cauliflower Mosaic Virus

CC-NB-LRR: coiled-coil nucleotide-binding leucine-rich repeat

cDNA: complimentary DNA

CE: collision energy

CFA: coronafacic acid

cfu ld^{-1}: colony forming units per leaf disc

cfu ml^{-1}: colony forming units per ml

CMA: coronamic acid

CNL: CC-NB-LRR

CO: Constans

COI1: Coronatine Insensitive1

Col-0: Columbia-0

cRNA: complimentary RNA

C_T: threshold cycle

CTR1: Constitutive Triple Response1

CUCs: Cup-shaped Cotyledons

CUL1: Cullin1

CXP: collision cell exit potential

CYP: Cytochrome P450

D: Deterium

D4SA: deuterium labeled SA

D5IAA: deuterium labeled IAA

D6ABA: deuterium labeled ABA

DNA: deoxyribonucleic acid

DNase: deoxyribonuclease

DP: declustering potential

dpi: days post inoculation

DT: dwell time monitoring

E: efficiency rate

EBF: EIN3-binding Factor

ECR1: E1 C-terminal Related1

EDR1: Enhanced Disease Resistance1

EDS: Enhanced Disease Susceptibility

EF-Tu: elongation factor-Tu

EFR: elongation factor-Tu (EF-Tu) receptor

EIN: Ethylene Insensitive

EP: collision cell exit potential

ERF1: Ethylene Response Factor1

ERS: Ethylene Response Sensor

ET: ethylene

ETI: effector-triggered immunity

ETP: EIN2 Targeting Protein

ETR: Ethylene Response

F: forward primer sequence

FCA: Full-Length Active

FLC: Flowering Locus C

FLS2: Flagellin Sensing2

FT: Flowering Locus T

g L^{-1}: gram per liter

GA: gibberellic acid

GH3: Glucocorticoid Hormone3

h: hours

H2JA: dihydrojasmonic acid

hpi: hours post inoculation

HR: Hypersensitive Response

IAA: indole acetic acid

IAA-Asp: indole acetic acid aspartic acid

IAA-Glu: indole acetic acid glutamic acid

iap: important for the ARR pathway

ICS: Isochorismate Synthase

Ile: isoleucine

IPL: Isochorismate Pyruvate Lyase

ISR: induced systemic resistance

IWFs: intercellular washing fluids

JA: jasmonic acid

JA/ET: jasmonic acid/ethylene

JA-Ile: jasmonic acid isoleucine

JAR1: Jasmonic Acid Resistant1

JAZ: Jasmonate ZIM-domain

JIN1: Jasmonate Insensitive1

kan: kanamycin

KB: King's B

LA: linolenic acid

LB: lysogeny broth

LC: liquid chromatography

LC/MS/MS: liquid chromatography/mass spectroscopy/mass spectroscopy

LD: Luminidependens

LFY: Leafy

LOX2: lipoxygenase2

LRR-RK: leucine-rich repeat receptor kinase

MAPKKK: Mitogen-activated Protein Kinase Kinase Kinase

MeJA: methyl jasmonate

MeOH: methanol

MeSA: methyl SA

$MgCl_2$: magnesium chloride

min: minutes

MKK: Mitogen-activated Protein Kinase Kinase

ml: milliliter

mL min^{-1}: milliliter per minute

mM: millimolar

MOBIX: The Institute for Molecular Biology and Biotechnology

MPK: Mitogen-activated Protein Kinase

N-terminus: amino-terminus

NAC: No Apical Meristem Cup-shaped Cotyledons

NACRS: NAC recognition sequence

NB-LRR: leucine-rich repeat

NDR1: Non Race-Specific Disease Resistance1

ng g^{-1}: nanograms per gram

NIMIN: NPR1/NIM1-interacting Protein

NPR1: Non-expressor of PR Genes1

OPDA: 12-oxo-phytodienoic acid

OPR3: oxo-phytodienoic acid reductase3

PAD: Phytoalexin Deficient

PAL: Phenylalanine Ammonia Lyase

PAMPs: pathogen-associated molecular patterns

PBI: Plant Biotechnology Institute

PCR: polymerase chain reaction

PDF1.2: Plant Defensin1.2

pm: picomole

PP2C: Protein Phosphatase Type 2C

PR: Pathogenesis Related

PRRs: pattern recognition receptors

Pst: Pseudomonas syringae pv. tomato

PTI: PAMP-triggered immunity

pv: pathovar

PYR1: Pyrabactin Resistance1

Q1: selected m/z of the first quadruple

Q3: selected m/z of the third quadruple

qPCR: quantitative polymerase chain reaction

qRT-PCR: quantitative reverse transcription polymerase chain reaction

R: reverse primer sequence

R2: coefficient of correlation

rif: rifampicin

RIN4: RPM1-interacting protein4

R_n: normalized reporter signal

RNA: ribonucleic acid

ROS: reactive oxygen species

RPM1: Resistance to Pseudomonas Maculicola1

RPS2: Resistance to Pseudomonas Syringae2

RT-PCR: Reverse Transcription Polymerase Chain Reaction

RUB: Related To Ubiquitin

SA: salicylic acid

SABPs: SA-binding proteins

SAG: SA glucoside

SAG13: Senescence Associated Gene13

SAH: Salicylate Hydroxylase

SAM: S-adenosyl-L-methionine

SAR: systemic acquired resistance

SCF: SKP1, Cullin, and F-box proteins

SD: standard deviation

sid2: salicylic acid induction deficient2

SOC1: Suppressor of Overexpression of Constans1

SnRK2: SNF-Related Protein Kinase2

START: Star-related Lipid-Transfer

SVP: Short Vegetative Period

TIR1: Transport Inhibitor Response1

TIR-NB-LRR: Drosophila Toll and mammalian interleukin (IL)-1 receptors

TMV: Tobacco Mosaic Virus

TNL: TIR-NB-LRR

Trp: tryptophan

TTSS: type III secretion system

UBQ: ubiquitin

un: untreated

V: volts

v:v: volume per volume

VSP1: Vegetative Storage Protein1

wpg: weeks post germination

Ws-2: Wassilewskija-2

Chapter 1:

Introduction

1.1 Preface

This chapter was written mostly by F. Al-Daoud, and some contributions were made by Dr. R.K. Cameron. It provides the reader with background information relevant to this thesis.

1.2 Plant defense

Plants are sessile organisms. Since they cannot flee from their predators, they have evolved a number of defense mechanisms to ensure their survival. Plants are attacked by a variety of organisms, including herbivorous animals, insects, fungi, bacteria, viruses, and parasitic plants.

1.2a Basal resistance/PAMP-triggered immunity (PTI)

Plants utilize a number of strategies to combat pathogens. One of their first responses to pathogen attack includes basal resistance or PAMP-triggered immunity (PTI) (Boller and He, 2009). PAMPs (pathogen-associated molecular patterns) are general features common to a variety of microbes. For example, a number of bacteria possess similar membrane lipopolysaccharides (Erbs and Newman, 2003). Plants have developed pattern recognition receptors (PRRs) that enable them to detect PAMPs and initiate PTI signaling (Boller and Felix, 2009). This results in a number of local physiological, biochemical, and molecular changes in the plant that are thought to slow the spread of infection (Boller and Felix, 2009; Boller and He, 2009; Nicaise et al., 2009).

The flagellin protein (a PAMP) that constitutes the flagellum of *Pseudomonas syringae* pv. *tomato* (*Pst*) is recognized by Flagellin Sensing2 (FLS2) of Arabidopsis (*Arabidopsis thaliana*). FLS2 is a leucine-rich repeat receptor kinase (LRR-RK) that

recognizes a 22 amino acid-long peptide at the N-terminus of flagellin called flg22 (Gomez-Gomez and Boller, 2000). Other Arabidopsis PRRs include the elongation factor-Tu (EF-Tu) receptor (EFR). EFR recognizes elf18, an 18 amino acid-long peptide at the N-terminus of EF-Tu of *Pst* (Kunze et al., 2004; Zipfel et al., 2006). Downstream of FLS2 and EFR is the LRR receptor-like kinase (LRR-RLK) Brassinosteroid-associated Kinase1 (BAK1: Chinchilla et al., 2007; Heese et al., 2007). The data suggests that BAK1 binds FLS2 and subsequently initiates PTI signaling (Chinchilla et al., 2007; Heese et al., 2007).

The FLS2 signaling pathway includes a number of kinase proteins: Mitogen-activated Protein Kinase6 (MPK6) is a positive regulator of PTI (Asai et al., 2002; Menke et al., 2004), whereas MPK4 is a negative regulator of PTI (Brodersen et al., 2006; Suarez-Rodriguez et al., 2007). These kinases are believed to regulate the activity of a number of proteins, including WRKY transcription factors (Pandey and Somssich, 2009) that control defense gene expression during PTI (Pitzschke et al., 2009). For example, MPK4 is thought to inhibit the transcriptional activity of WRKY33 by interacting with it in uninfected tissue and not allowing it to up-regulate expression of its regulon. Upon treatment with flg22, WRKY33 dissociates from MPK4 and induces expression of some defense genes (Qiu et al., 2008).

Treatment of plants with flg22 and/or elf18 results in a number of physiological, biochemical, and molecular changes. These include cytosolic calcium spikes (Aslam et al., 2009), reactive oxygen species (ROS) production (Gomez-Gomez et al., 1999; Aslam et al., 2009), intercellular space alkalinization (Bauer et al., 2001; Kunze et al., 2004),

increased ethylene (ET) (Bauer et al., 2001; Kunze et al., 2004; Navarro et al., 2004; Zipfel et al., 2006) and salicylic acid (SA) production (Tsuda et al., 2008; Wang et al., 2009), and callose deposition (Gomez-Gomez et al., 1999). A number of defense genes are up-regulated during PTI, including SA-associated genes, such as *Pathogenesis Related1* (*PR1*), jasmonic acid/ethylene (JA/ET)-associated genes, such as *Plant Defensin1.2* (*PDF1.2*), and *PR5* which is associated with JA/ET and SA signaling (Gomez-Gomez et al., 1999; Denoux et al., 2008; Aslam et al., 2009; Wang et al., 2009). These events are associated with increased resistance to pathogens (Kunze et al., 2004; Zipfel et al., 2006; Tsuda et al., 2008).

1.2b Bacterial virulence

A number of pathogens appear to have evolved mechanisms to inhibit PTI. For example, virulent *Pst* DC3000 (*Pst*) produces avirulent (Avr) protein effectors that are delivered into the plant cell via a type III secretion system (TTSS: a needle-like structure used by *Pst* to deliver effectors into host cells: Boller and He, 2009). Although many effectors promote susceptibility to *Pst*, they were named avirulence proteins because the first ones discovered are recognized by plants and induce resistance thereby reducing the virulence of *Pst* (discussed in the next section). *Pst* is predicted to possess up to 40 effectors (Guttman et al., 2002; Fouts et al., 2002; Petnicki-Ocwieja et al., 2002; Schechter et al., 2006). Many of them contribute to negative regulation of PTI by interacting with plant defense signaling proteins. Studies on AvrPto and AvrPtoB, for example, suggest that they bind to FLS2 and thereby reduce downstream PTI signaling (Gohre et al., 2008; Xiang et al., 2008). Also, HopAI1 seems to inhibit MPK6 activity

downstream of PTI receptors (Zhang et al., 2007). These strategies lead to decreased defense responses, including attenuated ROS production, callose deposition, and expression of some defense genes (Hauck et al., 2003; Zhang et al., 2007), thus culminating in a compatible interaction between Arabidopsis and *Pst* (i.e. *Pst* is able to colonize Arabidopsis in high numbers).

Furthermore, *Pseudomonas* exploits the relationship between different phytohormones to indirectly inhibit defense responses. For example, JA, ET, abscisic acid (ABA), and auxin are usually implicated in negative regulation of SA-associated responses, which promote resistance against *Pseudomonas* (discussed in section 1.3f) (Bari and Jones, 2009; Grant and Jones, 2009). *Pseudomonas* attempts to repress SA-related defenses by elevating *in planta* JA, ET, ABA, and auxin levels and signaling. AvrPto and AvrPtoB, for example, appear to up-regulate expression of some ET biosynthesis genes (Cohn and Martin, 2005). Also, AvrPtoB is implicated in promoting ABA accumulation (de Torres-Zabala et al., 2007), and AvrRpt2 contributes to positive regulation of auxin accumulation (Chen et al., 2007). Furthermore, some *Pseudomonas* bacteria produce their own ET and auxin (Weingart, et al., 2001; Ali et al., 2009), and some pathogens produce their own ABA (Crocoll et al., 1991). Moreover, almost half of the plant genes (42%) that are expressed in response to *Pst* effectors are associated with ABA signaling (de Torres-Zabala et al., 2007). Collectively, this leads to increased JA/ET, ABA, and auxin signaling, attenuated SA signaling, and increased susceptibility to *Pseudomonas*.

Some *Pseudomonas* strains also produce a number of phytotoxins that impair plant defense responses, such as coronatine (Bender et al., 1999). Coronatine consists of two moieties, coronafacic acid (CFA) and coronamic acid (CMA) that mimic JA and 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene, respectively (Feys et al., 1994; Ferguson and Mitchell, 1985). *Pseudomonas* uses coronatine to facilitate infection of Arabidopsis by up-regulating JA/ET signaling. This results in the opening of Arabidopsis stomata, inhibition of SA defense pathways, and promotion of susceptibility to *Pseudomonas* (Ferguson and Mitchell, 1985; Zhao et al., 2003; He et al., 2004; Block et al., 2005; Brooks et al., 2005; Melotto et al., 2006; Katsir et al., 2008; Melotto et al., 2008).

1.2c Resistance (*R*) gene-mediated resistance/effector-triggered immunity (ETI)

To counter inhibition of defenses by pathogens, plants are thought to have evolved resistance (*R*) gene-mediated resistance or effector-triggered immunity (ETI). ETI occurs when a plant that is expressing the appropriate *R* gene is infected with a pathogen expressing its cognate effector (Bent and Mackey, 2007; Boller and He, 2009). For example, *Pst* strains that possess the AvrRpt2 effector (*Pst avrRpt2*) trigger ETI in Arabidopsis plants that express the gene product of the *Resistance to Pseudomonas Syringae2* (*RPS2*) *R* gene (Whalen et al., 1991; Kunkel et al., 1993; Yu et al., 1993; Dangl and Jones, 2001). However, if a plant lacking an *R* gene is infected with a pathogen expressing its paired Avr effector, then the plant exhibits susceptibility. The idea that ETI occurs between a plant and a pathogen carrying corresponding *R* and *Avr* genes was first articulated in the gene-for-gene model (Flor, 1942).

Some R proteins directly interact with Avr effectors (Deslandes et al., 2003). However, many R proteins perceive the presence of Avr proteins in an indirect manner (Axtell et al., 2003; Axtell and Staskawicz, 2003; Mackey et al., 2003). RPS2, for example, does not directly interact with AvrRpt2. Rather, AvrRpt2 cleaves RIN4 (RPM1-interacting protein4) (Kim et al., 2005; Quirino and Bent, 2003), and RIN4 degradation is thought to be detected by RPS2 (the 'guard'). The indirect detection of AvrRpt2 by RPS2 initiates ETI signaling and results in increased resistance (Axtell et al., 2003; Axtell and Staskawicz, 2003; Mackey et al., 2003). This gave rise to the guard hypothesis, which postulates that Avr effectors modify plant proteins that are 'guarded' by R proteins (van der Biezen and Jones, 1998).

Many R proteins possess a nucleotide-binding site and a leucine-rich repeat (NB-LRR). These proteins are subdivided into two groups: one group contains a domain homologous to the intracellular signaling domains of the *Drosophila* Toll and mammalian interleukin (IL)-1 receptors (TIR-NB-LRR: TNL), and the other contains coiled-coil domains (CC-NB-LRR: CNL) (Dangl and Jones, 2001). TNL receptors and CNL receptors activate different pathways upon recognition of Avr effectors. CNL receptors, such as RPS2, activate a signaling pathway that is dependent on NDR1 (Non Race-Specific Disease Resistance1), whereas TNL receptors, such as RPS4, activate an EDS1 (Enhanced Disease Susceptibility1)-dependent pathway (Aarts et al., 1998). EDS1 is a lipase-like protein that is central to SA-associated defenses (Wiermer et al., 2005), and NDR1 is a membrane-bound protein (Century et al., 1997). Interestingly, NDR1 has been shown to interact with RIN4 (Day et al., 2006). It is hypothesized that NDR1

positively regulates defense responses by sequestering some RIN4 proteins thereby activating RPS2 and RPM1 (Day et al., 2006). Furthermore, RIN4 seems to be a negative regulator of PTI, as *rin4* plants exhibit increased resistance to virulent *Pseudomonas syringae* pv. *tomato* DC3000 (Mackey et al., 2002). It is hypothesized that *Pseudomonas* evolved AvrRpt2 and AvrRpm1 to take advantage of this fact and activate RIN4, thus increasing plant susceptibility. Arabidopsis may have subsequently evolved RPS2 and RPM1 to monitor this interaction and initiate ETI (Chisholm et al., 2006).

ETI signaling leads to a battery of plant defense responses including accumulation of reactive oxygen species (ROS) and SA, and up-regulation of some *PR* gene expression. This culminates in local programmed cell death, known as the Hypersensitive Response (HR). HR is thought to slow the spread of infection of plants by pathogens and lead to resistance (Dangl and Jones, 2001).

1.2d Systemic defense responses

Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are systemic defense responses that are initiated as a result of microbial infection. SAR and ISR involve production of a long distance signal that primes distal tissue and allows them to become resistant to a subsequent infection by a normally virulent pathogen (Durrant and Dong, 2004; Conrath et al., 2006). One difference between these two responses is that SAR is initiated in leaves after infection with a death-causing pathogen, while ISR is initiated in roots by nonpathogenic rhizobacteria. Long distance signaling during SAR and ISR (Champigny and Cameron, 2009) is associated with a number of plant hormones including SA, JA, and ET (van Bel and Gaupels, 2004; Chaturvedi et al., 2008; Delaney

et al., 1994; Forouhar et al., 2005; Metraux et al., 1990; Park et al., 2007; Schuhegger et al., 2006; Wildermuth et al., 2001; Yalpani et al., 2001). Lipid transport is also implicated in SAR (Maldonado et al., 2002). These signals lead to priming of distal tissue that includes up-regulation of some defense gene expression (Conrath et al., 2006).

1.3 The role of phytohormones in plant defense

Crosstalk between phytohormone signaling pathways during pathogen defense is complex. Much of the literature paints a picture where SA signaling usually promotes defense responses against biotrophic (require a living host to complete their lifecycle) and hemibiotrophic (require a living host for parts of their lifecycle) pathogens and inhibits defense responses against necrotrophic (live on dead organic matter) pathogens. In contrast, JA, ET, ABA, and auxin signaling usually positively regulate defense responses against necrotrophic pathogens and contribute to negative regulation of defense responses against (hemi)biotrophic pathogens (Fig 1.1) (Bari and Jones, 2009; Grant and Jones, 2009). When plants encounter a pathogen, they produce different levels of hormones depending on the kind of pathogen to which they are responding (De Vos et al., 2005). This is thought to allow the correct response to be initiated in an attempt to resist the invading microorganism.

1.3a Salicylic acid

Salicylic acid (SA) is a phenolic compound that regulates many processes in plants, including germination, growth, respiration, thermogenesis, flowering, and responses to abiotic and biotic stresses (Vlot et al., 2009). SA is produced *in planta* from

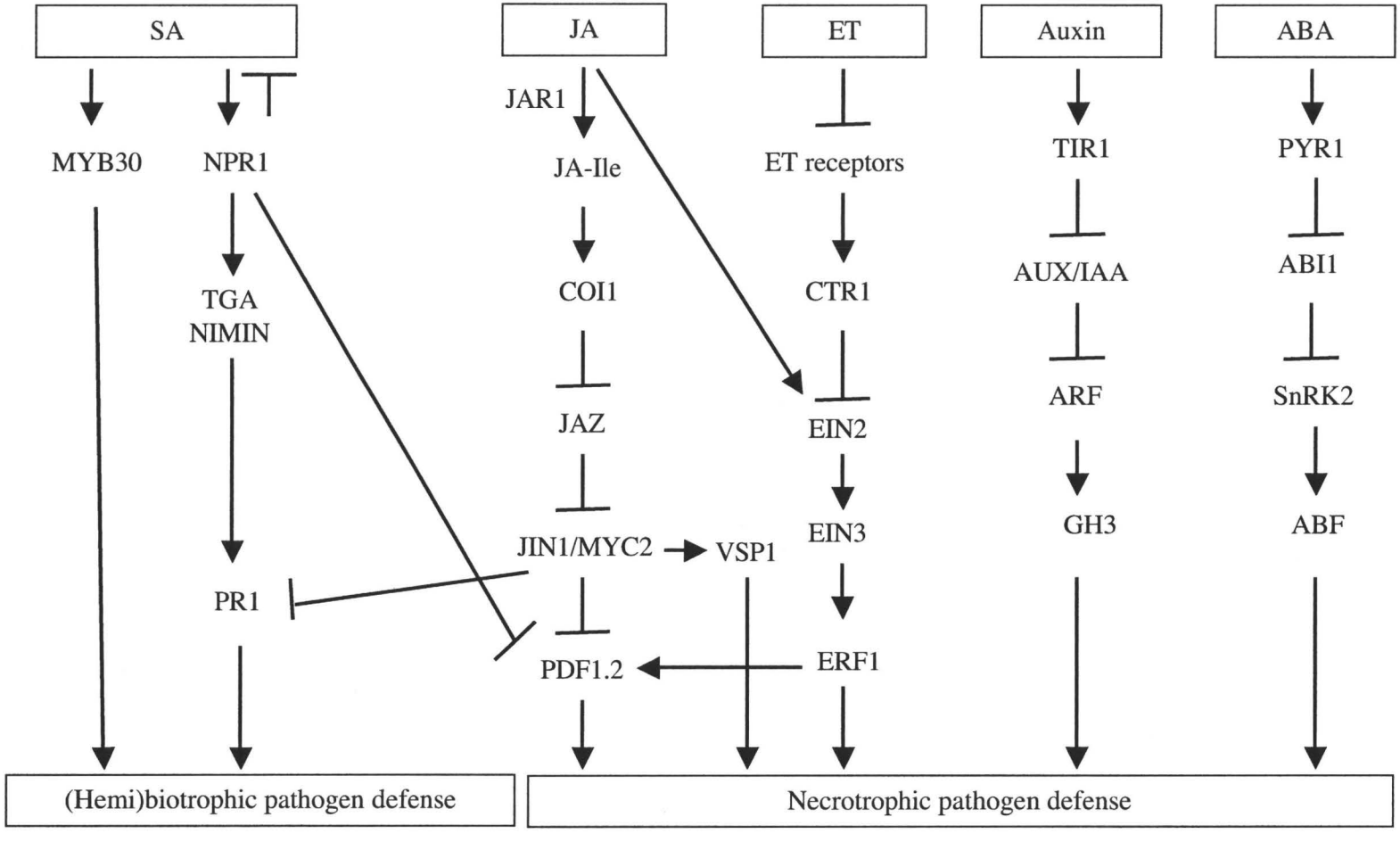


Figure 1.1. Diagram of the signaling pathways initiated by salicylic acid (SA), jasmonic acid (JA), ethylene (ET), auxin, and abscisic acid (ABA) during pathogen defense in Arabidopsis. SA mostly contributes to defense against (hemi)biotrophic pathogens, whereas JA, ET, auxin, and ABA mainly play a role in defense against necrotrophic pathogens. See text for details. → indicates positive regulation, ⊣ indicates negative regulation

chorismate in two main ways: 1) chorismate undergoes modification by chorismate mutase and a subsequent decarboxylation step to produce phenylalanine. Then a β-oxidation pathway that includes phenylalanine ammonia lyase (PAL) converts phenylalanine into benzoic acid (BA). Hydroxylation of BA produces SA. 2) Chorismate is converted into isochorismate by isochorismate synthase (ICS), which is then used by isochorismate pyruvate lyase (IPL) to make SA (Wildermuth, 2006). In Arabidopsis, the majority (~90%) of stress-induced SA is produced via the ICS pathway (Wildermuth et al., 2001). SA is stored as a conjugate of glucose, SA glucoside (SAG: Hennig et al., 1993; Song et al., 2008), or conjugated to amino acids (Staswick et al., 2002). Glucocorticoid Hormone3 (GH3) proteins contribute to the production of some SA-amino acid conjugates (Staswick et al., 2002). SA can also be found in its methylated form, methyl SA (MeSA: Park et al., 2007). MeSA has been implicated in systemic signaling during SAR in tobacco (Park et al., 2007), and plants infected with pathogens are thought to produce MeSA as an airborne signal to induce defense responses in nearby plants (Shulaev et al., 1997).

A number of SA-binding proteins (SABPs) have been identified in tobacco: SABP1 is a catalase (Chen et al., 1993), SABP2 is a MeSA esterase (Park et al., 2007), and SABP3 is a carbonic anhydrase (Slaymaker et al., 2002). SABP2 has the highest affinity for SA (Park et al., 2007). Studies suggest that SA produced in SAR-induced tobacco leaves inhibits the MeSA esterase activity of SABP2, and this is associated with elevated levels of MeSA and SAR signaling (Park et al., 2007). Likewise, SA appears to inhibit the ability of SABP1 and SABP3 to degrade and scavenge reactive oxygen species

(ROS) after infection with pathogens, which is associated with accumulation of ROS and manifestation of the HR (Chen et al., 1993, Dempsey et al., 1999). This suggests a link between SA perception, ROS accumulation, and programmed cell death.

Upstream of SA accumulation are Enhanced Disease Susceptibilty1 (EDS1) and Phytoalexin Deficient4 (PAD4). EDS1 and PAD4 are lipase-like proteins that interact with one another in plant leaves inoculated with pathogens (Zhou et al., 1998; Falk et al., 1999; Feys et al., 2001). They are required for SA production during PTI and ETI, and they appear to be positively regulated by SA via a feedback loop (Wiermer et al., 2005). MPK4 and Enhanced Disease Resistance1 (EDR1), a MAPKKK (mitogen-activated kinase kinase kinase), are implicated in negatively regulating SA signaling by inhibiting EDS1/PAD4. This is demonstrated by data that suggests that *mpk4* and *edr1* mutant plants exhibit elevated EDS1/PAD4-dependent SA production (Frye et al., 2001; Brodersen et al., 2006). Some MPKs also seem to be involved in regulating SA signaling downstream of SA production. MPK6 and to a lesser extent MPK3 are required for SAR in Arabidopsis and a priming event that occurs after treating Arabidopsis with a SA analogue, suggesting that MPK3 and MPK6 are positive regulators of SA signaling (Beckers et al., 2009).

SA plays a key role during SAR, as SA treatment up-regulates many of the same genes induced during SAR, and SA accumulation- and SA biosynthesis-deficient plants are not able to mount a successful SAR response (Ryals et al., 1996). A key regulator of SA signaling during SAR is the transcriptional activator Non-expressor of PR Genes1 (NPR1: Dong, 2004). NPR1 contains an ankyrin-repeat motif and a BTB/POZ (broad-

complex tramtrack bric-a-brac poxvirus and zinc finger) domain that participate in protein-protein interactions (Durrant and Dong, 2004; Pieterse and Van Loon, et al., 2004). NPR1 interacts with and apparently activates bZIP (basic leucine zipper) transcription factors (members of the TGA family). It also interacts with a number of other proteins, including NPR1/NIM1-interacting (NIMIN) 1, 2, and 3 (Weigel et al., 2005; Rochon et al., 2006; Shearer et al., 2009). Some TGA transcription factors seem to inhibit SA-related defense gene expression, whereas others appear to promote it (Kesarwani et al., 2007). TGA proteins are also thought to be regulated by NIMIN proteins after it was observed that NIMIN proteins interact with NPR1-TGA complexes (Weigel et al., 2005).

In addition to NPR1-dependent gene expression, SA also activates NPR1-indpendent pathways. For example, a MYB30 transcription factor that is involved in the HR in response to an avirulent strain of *Pseudomonas* is expressed in response to SA in a NPR1-independent manner. Furthermore, MYB30 does not appear to require NPR1 to induce expression of its regulon (Fig 1.1) (Raffele et al., 2006). Additionally, a WHIRLY transcription factor found to be required for some SA-associated gene expression does not require NPR1 to bind its target DNA sequence (Desveaux et al., 2004; Raffaele et al., 2006). NPR1-independent signaling seems to contribute to early responses to SA (within 30 min of SA treatment), whereas NPR-dependent signaling appears to regulate the majority of later response to SA (Blanco et al., 2005; Thurow et al., 2005).

1.3b Jasmonic acid

Jasmonic acid (JA) is involved in cell growth, and biotic and abiotic stress responses (Kazan and Manners, 2008). JA is synthesized *in planta* by converting the fatty acid linolenic acid (LA) into 13-hydroperoxylinolenic acid. Lipoxygenase2 (LOX2) is required for this conversion (Bell et al., 1995; Bell and Mullet, 1993). Then, allene oxide synthase (AOS), a cytochrome P450 (CYP), uses 13-hydroperoxylinolenic acid to produce the JA precursor 12-oxo-phytodienoic acid (OPDA: Laudert and Weiler, 1998; von Malek et al., 2002; Park et al., 2002). OPDA is reduced by an oxo-phytodienoic acid reductase (OPR3: Sanders et al., 2000; Stintzi and Browse, 2000), which is followed by three ß–oxidation steps to produce JA (Feussner and Wasternack, 2002; Kazan and Manners, 2008; Turner et al., 2002). LOX2, AOS, and OPR3 are all required for JA accumulation (Bell et al., 1995; Bell and Mullet, 1993; Sanders et al., 2000; Stintzi and Browse, 2000; Park et al., 2002; Spoel et al., 2003; Stenzel et al., 2003; Raacke et al., 2006).

JA is present in a number of different forms *in planta*, including methyl jasmonate (MeJA), and it can be conjugated to some amino acids including isoleucine (Ile) (Staswick et al., 2002; Staswick and Tiryaki, 2004). Similar to SA, JA is conjugated to amino acids by GH3 proteins, including Jasmonic Acid Resistant1 (JAR1) (Staswick et al., 2002). OPDA, JA, and JA derivatives are biologically active and regulate expression of some common target genes (McGrath et al., 2005; Sasaki-Sekimoto et al., 2005; Mueller et al., 2008; Wang et al., 2008). JA-Ile and its *Pst*-produced mimic coronatine, interact with the Coronatine Insensitive1 (COI1) component of the ubiquitin E3 ligase

SCF^{COI1} (SKP1, Cullin, and F-box proteins) (Katsir et al., 2008; Melotto et al., 2008). This interaction promotes an association between COI1 and members of the Jasmonate ZIM-domain (JAZ) family (Katsir et al., 2008; Melotto et al., 2008). Several JAZ proteins are directed to the proteasome for degradation in response to JA treatment in a manner that is dependent on ubiquitination by SCF^{COI1} (Chini et al., 2007; Thines et al., 2007). JAZ proteins are thought to function as negative regulators of JA signaling (Thines et al., 2007; Yan et al., 2007) by interacting with and inhibiting the activity a number of transcription factors, including the helix-loop-helix transcription factor Jasmonate Insensitive1 (JIN1). This is demonstrated by data that suggests that plants with a non-functional JAZ protein overexpress JIN1-regulated genes (Chini et al., 2007). JIN1 regulates expression of many JA-responsive genes such as *Vegetative Storage Protein1* (*VSP1*) and the JA/ET signaling marker gene *Plant Defensin1.2* (*PDF1.2*) (Fig 1.1) (Berger et al., 1996; Lorenzo et al., 2004; Dombrecht et al., 2007). This contributes to defense responses against a wide variety of necrotrophic pathogens.

1.3c Ethylene

Ethylene (ET) is involved in a number of plant development and stress response processes. These include seed germination, root development, flowering, senescence, and responses to biotic and abiotic stresses (Adie et al., 2007; Lin et al., 2009). ET treatment induces a number of plant immune responses, including phytoalexin production (Adie et al., 2007), cross-linking of plant cell wall proteins which may increase fortification (Toppan et al., 1982), and production of obstruction gels in plant vasculature thought to contribute to sealing up damaged plant tissue (VanderMolen et al., 1983). ET positively

regulates defense against necrotrophs and negatively regulates defense responses to biotrophic and hemibiotrophic pathogens. The ethylene signaling mutant *ethylene insensitive2* (*ein2*: Alonso et al., 1999), for example, exhibits increased resistance to *Pseudomonas* (Bent et al., 1992; Chen et al., 2009) and increased susceptibility to the fungus *Botrytis cinerea* (Thomnma et al., 1999).

ET is produced from methionine. Methionine is converted into *S*-adenosyl-*L*-methionine (*S*-AdoMet: SAM) by SAM synthase. ACC synthase produces ACC (1-aminocyclopropane-carboxylic acid) from SAM. This is considered to be the rate-limiting step for ET production. Finally, ET is generated from ACC by ACC oxidase (ACO) (Adie et al., 2007; Lin et al., 2009).

ET is thought to be perceived by a family of receptors, including ETR1 (Ethylene Response1), ETR2, ERS (Ethylene Response Sensor), ERS2, and EIN4 (Ethylene Insensitive4), which are similar to bacterial two-component histidine kinases (Bleeker et al., 1988; Chang et al., 1993; Hua and Meyerowitz, 1998). Data suggests that in the absence of ET, these receptors inhibit ET signaling (Hua and Meryerowitz, 1998) by activating downstream components that negatively regulate ET signaling, including the protein kinase Constitutive Triple Response1 (CTR1) (Kieber et al., 1993). ET is thought to deactivate the receptors leading to alleviation of the negative regulation, which in turn activates ET signaling. Ethylene Insensitive2 (EIN2) is an important component of the ET signaling pathway downstream of the ET receptors and CTR1 (Hall and Bleecker, 2003). The EIN2 protein is thought to be similar to members of the Nramp family of metal transporters (Alonso et al., 1999). The biochemical function of EIN2, however,

remains to be determined. Downstream of EIN2, the EIN3 transcription factor activates a transcription factor cascade, including expression of the *Ethylene Response Factor1* (*ERF1*) transcription factor. ERF1 is involved in up-regulating expression of some JA/ET-responsive genes (Fig 1.1) (Solano et al., 1998). EIN2 and EIN3 proteins have short half-lives in the absence of ET (Guo and Ecker, 2003; Qiao et al., 2009). This is associated with *in vivo* interaction between EIN2 and two F-box proteins (EIN2 Targeting Protein1 (ETP1) and ETP2) and interaction between EIN3 and two F-box proteins (EIN3-binding Factor1 (EBF1) and EBF2). These F-box proteins are implicated in proteasome-mediated degradation of EIN2 and EIN3. When ET production is induced, however, accumulation of some of these F-box proteins, such as ETP1 and ETP2, is reduced and EIN2 and EIN3 protein levels are elevated (Guo and Ecker, 2003; Qiao et al., 2009).

1.3d Auxin

Auxin (indole acetic acid, IAA) is involved in many aspects of plant development (Woodward and Bartel, 2005). Recently, auxin has been shown to regulate plant defense responses against pathogens (Bari and Jones, 2009; Grant et al., 2009; Kazan and Manners, 2009). For example, the auxin signaling mutant *auxin resistant2* (*axr2*) exhibits increased resistance to the hemibiotrophic *Pseudomonas* bacterium (Wang et al., 2007), and *axr1*, *axr2*, and *axr6* exhibit increased susceptibility to the necrotrophic *Plectosphaerella curumerina* and *Botrytis cinerea* fungi (Llorente et al., 2008). Also, increased resistance to *Pseudomonas* and the biotrophic oomycete *Hyaloperonospora arabidopsidis* is observed in plants that overexpress transcripts that encode for a negative

regulator of auxin signaling, Mitogen-activated Protein Kinase Kinase7 (MKK7) (Zhang et al., 2007). Therefore, this evidence suggests that auxin signaling positively regulates defense responses against some necrotrophic pathogens and negatively regulates defense responses against some biotrophs and hemibiotrophs (Bari and Jones, 2009; Grant et al., 2009; Kazan and Manners, 2009).

Arabidopsis produces auxin via tryptophan (Trp)-dependent and Trp-independent pathways (Woodward and Bartel, 2005). Auxin occurs in many forms *in planta*, including free auxin and conjugated auxin. Auxin can be conjugated to sugars, amino acids, and proteins (Kleczkowski and Schell, 1995). Similar to JA and SA, auxin is conjugated to amino acids by GH3 proteins (Staswick et al., 2005). The majority of auxin in Arabidopsis (~90%) is in the conjugated form (Tam et al., 2000). However, free auxin is the only known active form of auxin, whereas conjugated auxin plays an important role in auxin storage (LeClere et al., 2002). Some IAA conjugates, including IAA-Asp and IAA-Glu, are not used to store IAA, rather they are thought to be intermediates in a pathway that leads to IAA catabolism (Ljung et al., 2002). Therefore, plants seem to alter the level of free auxin by diverting it to storage, and/or marking it for degradation.

Auxin binds to an F-box receptor protein known as Transport Inhibitor Response1 (TIR1: Dharmasiri et al., 2005, Kepinski and Leyser, 2005). TIR1 is a component of a SCF^{TIR1} complex that interacts with repressors of auxin signaling, such as members of the AUX/IAA protein family (Gray et al., 2001). TIR1 is required for auxin-induced degradation of AUX/IAA, which is thought to occur via the ubiquitin/26S proteasome pathway after ubiquitination of AUX/IAA proteins by the auxin-SCF^{TIR1} complex (Gray

et al., 2001). This alleviates inhibition of auxin signaling and allows Auxin Response Factor (ARF) transcription factors to up-regulate expression of some auxin-responsive genes, such as *GH3* genes (Fig 1.1) (Guilfoyle et al., 1998). It has been speculated that AUX/IAA proteins negatively regulate auxin signaling by inhibiting ARF proteins (Liscum and Reed, 2002).

1.3e Abscisic acid

Abscisic acid (ABA) is a terpenoid phytohormone derived from the plastidal 2-C-methyl-d-erythritol-4-phosphate pathway. ABA is biologically active in its free form, and it can be transported to different parts of the plant as an inactive glucose conjugate (Wasilewska et al., 2008; Ton et al., 2009). ABA negatively regulates a number of plant development processes, including seed germination, and it promotes many abiotic stress responses, such as water deficit stress tolerance (Wasilewska et al., 2008; Ton et al., 2009).

A number of ABA-binding proteins have been identified in plants, including G-protein-coupled receptors (Liu et al., 2007; Pandey et al., 2009), the RNA-binding Full-Length Active (FCA) protein (Razem et al., 2006), the H subunit of a chelatase complex (Shen et al., 2006), and Pyrabactin Resistance1 (PYR1), a member of the Star-related Lipid-Transfer (START) protein family (Iyer et al., 2001; Park et al., 2009). Signaling that occurs when ABA binds these putative receptors is not well understood. However, after ABA binds PYR1, PYR1 is thought to deactivate Protein Phosphatase Type 2C (PP2C: Park et al., 2009). PP2C proteins, including ABA Insenstive1 (ABI1), negatively regulate ABA signaling (Sheen, 1998) possibly by deactivating positive regulators such

as SNF-Related Protein Kinase2 (SnRK2: Park et al., 2009). Research suggests that SnRK2 proteins phosphorylate and thereby activate ABA-responsive transcription factors, including ABA Response Element Binding Factors (ABFs), which induce expression of a number of ABA-related genes (Fig 1.1) (Fujii et al., 2007). Therefore, ABA appears to elicit plant responses by derepressing ABA signaling. This is similar to the mode of action of JA, ET, and auxin discussed above.

During infection by pathogens, *in planta* ABA levels increase in response to a number of biotrophs, hemibiotrophs, and necrotrophs (Whenham et al., 1986; Kettner and Dorffling, 1995; de Torres-Zabala et al., 2007). ABA plays a variety of roles during resistance to pathogens depending on the stage of infection and the pathosytem. Treatment of some plants with ABA results in increased resistance to Tobacco Mosaic Virus (TMV: Flors et al., 2005), and the necrotrophic fungi *Botrytis cinerea* (Audenaert et al., 2002) and *Alternaria brassicicola* (Flors et al., 2008). Conversely, ABA treatment of some plants results in increased susceptibility to hemibiotrophic *Pst* (de Torres-Zabala et al., 2007; Yasuda et al., 2008), and the necrotrophic fungus *Phytophthora megasperma* (Ward et al., 1989).

In the Arabidopsis-*Pst* system ABA enhances the ability of *Pst* to colonize Arabidopsis during late stages of infection, but it inhibits the ability of *Pst* to infect Arabidopsis during early stages of infection. For example, perception of the *Pst* PAMP flg22 peptide by the Arabidopsis FLS2 receptor induces an ABA and SA signaling-dependent response that results in closure of the stomatal pores of Arabidopsis leaves (Melotto et al., 2006). This reduces the ability of *Pst* to access the stomatal openings and

colonize the intercellular space of these leaves. However, later stages of resistance to *Pst* are negatively regulated by ABA if the aforementioned early stage of infection is circumvented by injecting *Pst* directly into the intercellular space of Arabidopsis leaves. A number of ABA biosynthesis-deficient mutants, for example, exhibit enhanced resistance to *Pst* which is associated with increased callose deposition and increased SA-associated defense responses compared to wild-type plants (de Torres-Zabala et al., 2007; de Torres Zabala et al., 2009). In contrast, ABA hypersensitive mutants support increased *Pst* growth associated with decreased callose deposition (de Torres-Zabala et al., 2007). Also, exogenous application of ABA onto Arabidopsis is associated with inhibition of a number of defense responses including callose deposition, SA accumulation, and expression of some SA- and JA/ET-associated genes after inoculation with *Pst* (Mohr and Cahill, 2007). ABA signaling also seems to inhibit SAR against *Pst* by down-regulating SA biosynthesis and SA signaling (Yasuda et al., 2008). This data suggests that ABA signaling negatively regulates resistance to *Pst* by curbing callose deposition and SA-related defense responses.

Contrary to the data presented above, ABA accumulation is also associated with enhanced callose deposition during priming of Arabidopsis against some fungal infection. Priming occurs when plants are exposed to biotic or abiotic factors that result in a physiological state that allows the plant to mount a stronger and more effective defense response against a subsequent pathogen attack (Conrath et al., 2006). For example, treatment of Arabidopsis with β-aminobutyric acid (BABA) is associated with enhanced resistance to a number of pathogens, including the fungus *Alternaria brassicicola* (Ton

and Mauch-Mani, 2004). This induced resistance response is accompanied by expression of some defense genes and callose deposition (Ton and Mauch-Mani, 2004), and it is associated with expression of some ABA biosynthesis genes and requires ABA accumulation (Flors et al., 2008). Therefore, whereas accumulation of ABA negatively regulates callose deposition during defense responses to *Pst*, it promotes callose deposition during BABA-induced resistance to *A. Brassicciola.*

1.3f Interaction between phytohormones during defense

In Arabidopsis, defense signaling pathways that are effective against (hemi)biotrophs often contribute to negative regulation of resistance to necrotrophs and visa versa. This is demonstrated by the observation that plants inoculated with hemibiotrophic *Pst* exhibit increased susceptibility to necrotrophic *Alternaria brassicicola* (Spoel et al., 2007). This seems to occur as a result of the mutually antagonistic relationship between SA, which is the key player in defense against (hemi)biotrophs, and JA, ET, and auxin, which promote defense against necrotrophs (Bari and Jones, 2009; Grant and Jones, 2009).

Many SA-associated transcription factors appear to inhibit expression of some JA/ET-related genes. For example, NPR1 and WRKY70 are implicated in up-regulating some SA-associated gene expression while negatively regulating expression of some JA/ET-associated genes (Spoel et al., 2003; Li et al., 2006). Components of JA/ET signaling, including COI1, JIN1, and ERF1 contribute to negative regulation of SA-mediated resistance to *Pst* by inhibiting SA accumulation and SA signaling (Fig 1.1) (Kloek et al., 2001; Berrocall-Lobo et al., 2004; Nickstadt et al., 2004). However, some

similarities exist between SA and ET signaling, as two related MAPKKK proteins, EDR1 and CTR1, negatively regulate SA- and ET-related responses, respectively (Frye et al., 2001).

The mutually antagonistic relationship between auxin and SA is illustrated by the observation that exposure of Arabidopsis to the SA analog benzothiadiazole-S-methyl ester (BTH) inhibits auxin transport, perception, and signaling (Wang et al., 2007). Also, reduced SA accumulation in *NahG* plants is associated with elevated levels of auxin relative to the wild-type (Abreu and Munne-Bosch, 2009). Auxin signaling also negatively regulates SA signaling, as treatment of Arabidopsis with auxin reduces SA-induced expression of *PR1* (Wang et al., 2007), and results in susceptibility to *Pseudomonas* (Chen et al., 2007). Furthermore, decreased auxin signaling in *MKK7*-overexpressing plants is associated with elevated SA levels, constitutive expression of *PR* genes, and enhanced resistance to *Pseudomonas* and *H. arabidopsidis* (Zhang et al., 2007). In contrast, auxin signaling seems to co-operate with JA/ET signaling during defense responses to necrotrophs. The auxin signaling mutant *axr1*, for example, exhibits reduced expression of some JA-associated genes, including *LOX2*, *AOS*, and *VSP* (Tiryaki and Staswick, 2002), and ET biosynthesis-associated genes, such as *ACC Synthase4* (*ACS4*) (Abel et al., 1995). This data suggests that auxin inhibits some SA-associated defense responses and enhances some JA/ET-associated defense responses.

The auxin and JA signaling pathways have many similarities. For example, auxin interacts with TIR1 and JA-Ile interacts with COI1, both of which are F-box protein components of SCF complexes. TIR1 and COI1 are thought to ubiquitinate and promote

degradation of negative regulators of auxin (AUX/IAA proteins) and JA (JAZ proteins) signaling, respectively. This appears to lead to derepression of the MYC2/JIN1 and ARF transcription factors and culminates in expression of some JA- and auxin-associated genes (Gray et al., 2001; Katsir et al., 2008; Melotto et al., 2008). Furthermore, both SCF complexes, SCF^{TIR1} and SCF^{COI1}, seem to be co-regulated. The Cullin1 (CUL1) protein present in both complexes (Hellmann et al., 2003; Ren et al., 2005) is activated by the RUB (Related To Ubiquitin) enzyme, which is composed of AXR1 (ubiquitin-like activating enzyme) and ECR1 (E1 C-terminal Related1) proteins (Hellmann and Estelle, 2002). A disruption in AXR1 or CUL1 affects both auxin and JA signaling (Tiryaki and Staswick, 2002; Hellmann et al., 2003; Ren et al., 2005; Kazan and Manners, 2009). Also, deactivation of ARF6, ARF8, or JIN1/MYC2 transcription factors disrupts both auxin and JA signaling (Nagpal et al., 2005; Dombrecht et al., 2007). Collectively, this data suggests that auxin and JA signaling are intimately linked.

ET seems to interact with JA and auxin in a synergistic manner (Adie et al., 2007; Lin et al., 2009). For example, expression of the JA/ET marker *PDF1.2* is dependent on components of ET and JA signaling (Fig 1.1) (Penninckx et al., 1998), and expression of some auxin biosynthetic enzymes is responsive to ET treatment (Stepnova et al., 2008). Also, ET and auxin signaling positively regulate each other during some developmental processes, such as root development (Negi et al., 2008; Rahman et al., 2008).

The interaction between ABA and other phytohormones is complex (Ton et al., 2009). ABA treatment reduces expression of some SA-associated genes in Arabidopsis (Mohr and Cahill, 2007; Yasuda et al., 2008), and some ABA biosynthesis mutants have

elevated SA levels, whereas SA accumulation-deficient *salicylic acid induction deficient2 (sid2)* plants accumulate less ABA than the wild-type after inoculation with *Pst* (de Torres Zabala et al., 2009). This suggests that ABA negatively regulates SA signaling and production, while SA positively regulates ABA biosynthesis.

Expression of the JA/ET signaling marker *PDF1.2* is up-regulated in some ABA biosynthesis mutants, whereas it is reduced in some ABA signaling mutants after MeJA treatment (Anderson et al., 2004). Also, ABA is required for JA biosynthesis in Arabidopsis after fungal infection (Adie et al., 2007), expression of *JIN1/MYC2* is responsive to ABA treatment, and JIN1/MYC2 appears to regulate expression of some ABA-associated genes (Abe et al., 1997, Abe et al., 2003; Anderson et al., 2004). This data suggests that ABA positively regulates JA biosynthesis and negatively regulates some downstream JA signaling, and JIN1/MYC2 regulates JA/ET and ABA signaling.

The *etr1* and *ein3* ethylene signaling mutants have elevated expression of some ABA-related genes (Anderson et al., 2004). ET treatment, however, induces ABA production (Hansen and Grossmann, 2000; Benschop et al., 2007), whereas ABA treatment quells ET production (LeNoble et al., 2004). This suggests that ABA inhibits ET synthesis, while ET has positive effects on ABA accumulation but negatively regulates downstream ABA signaling.

1.4 Age-related resistance

Resistance to infection by pathogens varies with plant age (Panter and Jones, 2002; Whalen, 2005; Develey-Riviere and Galiana, 2007). Resistance to some pathogens increases as some plants mature (Kim et al., 1989; Shaik et al., 1989a; Shaik et al.,

1989b; Hwang, 1995; Canals and Pinochet, 1992; Dickson and Petzoldt, 1993; Leisner et al., 1993; Yalpani et al., 1993; DiFonzo et al., 1994; Kift et al., 1996; Hugot et al., 1999; Kus et al., 2002; Chern et al., 2005; Rusterucci et al., 2005; Roumen et al., 1992; Montesinos et al., 1995; Sirjusingh et al., 1996; Giorcelli et al., 1996; Heitefuss et al., 1997; Salzman et al., 1998). Conversely, resistance to other pathogens decreases with increasing plant age (Shaik et al., 1989a; Shaik et al., 1989b; Dubey, 1997; Shtienberg et al., 1993). For example, as rice matures it becomes more resistant to *Xanthomonas oryzae* (Chern et al., 2005), whereas aging bean leaves become more susceptible to *Uromyces appendiculatus* (Shaik et al., 1989a; Shaik et al., 1989b).

Increased resistance to pathogens with plant age is known as age-related resistance (ARR). The onset of ARR in some species is associated with the transition from vegetative to reproductive growth, senescence, or stressful growing conditions (Panter and Jones, 2002; Whalen, 2005; Develey-Riviere and Galiana, 2007). As plants age they undergo many biochemical and molecular changes. For example, increased expression of some *PR* genes is observed in older tobacco leaves (Uknes et al., 1993, Yalpani et al., 1993), leaves of flowering tobacco plants (Herbers et al., 1996; Hugot et al., 1999; Hugot et al., 2004), and tobacco flowers (Lotan et al., 1989; Uknes et al., 1993).

As tobacco matures it exhibits ARR against *Peronospora tabacina* (Wyatt et al., 1991), *Phytophthora parasitica* (Hugot et al., 1999), and *Tobacco Mosaic Virus* (*TMV*: Fraser, 1981; Yalpani et al., 1991; Yalpani et al., 1993). This is associated with flowering, and accumulation of *PR* gene transcripts and anti-microbial compounds in

mature untreated plants (Fraser, 1981; Wyatt et al., 1991; Yalpani et al., 1993; Hugot et al., 1999; Hugot et al., 2004). Studies with *NahG* tobacco plants that accumulate little SA indicate that mature tobacco plants inhibit the initial infection by *P. parasitica* in a salicylic acid (SA)-independent manner, such that *NahG* and wild-type plants exhibit a similar decrease in symptoms to *P. parasitica* as they age. However, mature *NahG* plants exhibiting symptoms show a more pronounced expansion of fungal growth than wild-type plants, suggesting that tobacco plants utilize SA-dependent mechanisms to reduce the spread of *P. parasitica* after an initial infection during ARR (Hugot et al., 1999). Therefore, there are SA-dependent and SA-independent ARR pathways in tobacco.

ARR in some systems involves *R* genes (Whalen, 2005; Develey-Riviere and Galiana, 2007). For example, mature tomato plants expressing *Cf-9B* exhibit resistance to races of the biotrophic fungus *Cladosporium fulvum* that express the Avr9 effector (Panter et al., 2002). Cf-9B-mediated resistance is active in mature plants, but not in seedlings or in the vegetative growth stage. The onset of this ARR is thought to occur between the vegetative and fruiting stages of development (Panter et al., 2002). ARR in this system, however, is not associated with developmentally-regulated expression of *Cf-9B*. The promoter of *Cf-9B* is active in seedlings as well as mature plants. This suggests that expression of *Cf-9B* may be required for this ARR response, but it does not regulate the onset of this resistance response (Panter et al., 2002). In addition, some *R* gene products are similar to the structure of some proteins involved in development. For example, the *R* gene *Resistance to Peronospora Parasitica27* (*RPP27*) encodes for a

receptor-like protein that is similar to CLAVATA2, which is involved in meristem and organ development (Tor et al., 2004).

As Arabidopsis ages it becomes more resistant to virulent and avirulent *Pst* (Kus et al., 2002; Rusterucci et al., 2005), *Ps* pv. *maculicola* (Kus et al., 2002), *P. viridiflava* (*Pv*: Goss and Bergelson, 2006), the oomycete *Hyaloperonospora arabidopsidis* (formerly *Hyaloperonospora parasitica* and *Peronospora parasitica*) (McDowell et al., 2005; Rusterucci et al., 2005), and *Cauliflower Mosaic Virus* (*CaMV*: Leisner et al., 1993). However, young and mature Arabidopsis display similar levels of susceptibility to the necrotrophic bacterium *Erwinia carotovora* (Rusterucci et al., 2005). In addition, Arabidopsis becomes more susceptible to the necrotrophic fungus *Botrytis cinerea* and biotrophic fungus *Erysiphe cichoracearum* with age (Rusterucci et al., 2005). Overall, Arabidopsis exhibits ARR to some biotrophic and hemibiotrophic pathogens.

A number of changes occur in Arabidopsis as it matures. These include elevated levels of glucosinolates, which can be metabolized to produce toxins and anti-herbivory compounds, in leaves and roots of Arabidopsis as it matures from the seedling stage to after the transition to flowering (12 hr. photoperiod: Petersen et al., 2002). In addition, up-regulation of *PR2* and *PR5*, but not *PR1*, is observed in untreated, mature, six week-old plants (12 hr. photoperiod: Chen and Chen, 2002), and some defense genes are up-regulated in leaves undergoing senescence (Quirino et al., 1999). Also, the source-sink relationship between mature and young leaves changes, which may limit accessibility to certain plant tissue for pathogens that move via the vasculature such as *CaMV* (Leisner et al., 1993).

Similar to tobacco, ARR in Arabidopsis to some pathogens is associated with flowering. For example, when Arabidopsis is grown under long days it flowers and exhibits ARR to *Pst* earlier than when it is grown under short day conditions (Rusterucci et al., 2005). Furthermore, flowering delayed mutants are more symptomatic to CaMV inoculation than wild-type plants of the same age (Cecchini et al., 1993), whereas early flowering mutants and ecotypes display increased resistance to CaMV and *P. viridiflava* compared to plants of the same age that flower later (Leisner et al., 1993). This is attributed to accelerated onset of ARR in early flowering plants and delayed onset of ARR in plants that flower later (Leisner et al., 1993; Cecchini et al., 2002; Rusterucci et al., 2005; Goss and Bergelson, 2006). This suggests that the transition from vegetative to reproductive growth is important for the onset of ARR in Arabidopsis.

To determine if ARR to *Pst* is also associated with senescence, expression of the senescence marker gene *SAG13* (*Senescence Associated Gene13*) was measured in mature plants (Kus et al., 2002). *SAG13* was not detected in untreated, ARR-competent plants suggesting that ARR is not associated with senescence in this system.

Mature Arabidopsis (5 to 6 weeks post germination: wpg) support up to 100-fold less *in planta Pst* growth than young plants (3 wpg; Kus et al., 2002). This is a whole plant phenomenon, as both young and mature leaves become more resistant to *Pst* as Arabidopsis ages (Kus et al., 2002). ARR is distinct from a number of defense pathways including SAR and ISR, as the SAR-defective *non-expresser of PR genes1* (*npr1*) and the ISR-defective *ethylene receptor1* (*etr1*) exhibit ARR (Kus et al., 2002). *phytoalexin accumulation deficient3* (*pad3*), a camalexin accumulation-deficient mutant, and

enhanced disease susceptibility 7 (*eds7*), which is impaired in resistance to *Pst* and *Psm*, also exhibit ARR, suggesting that camalexin and EDS7 are not required for ARR (Kus et al., 2002). Furthermore, the JA signaling mutants *jin1* and *jar1* also exhibit ARR (Carviel et al., 2009). As a whole, this data suggests that NPR1, ETR1, PAD3, EDS7, JIN1, and JAR1 are not required for ARR.

ARR does not occur in a number of SA accumulation-deficient mutants, including *NahG*, *SA induction-deficient1* (*sid1*), *sid2*, *pad4*, *eds1*, and *important for the ARR pathway1-1* (*iap1-1*) (Kus et al., 2002; Cameron and Zaton, 2004; Carviel et al., 2009). *NahG* plants express a salicylate hydroxylase protein that converts SA into inert catechol (Gaffney et al., 1993; Vernooij et al., 1995), and *sid1* and *sid2* plants contain mutations in *EDS5*, a putative SA transporter (Nawrath et al., 2002), and *Isochorismate Synthase1* (*ICS1*), a SA biosynthesis gene (Wildermuth et al., 2001), respectively. PAD4 and EDS1 are lipase-like proteins that interact with one another and are required for SA accumulation during infection with *Pst* (Wiermer et al., 2005). Taken together, this data suggests that SA accumulation is necessary for ARR.

SA usually acts as a signal during resistance to pathogens to up-regulate expression of *PR* genes through activation of NPR1, as young *npr1* accumulates fewer *PR1* transcripts than the wild-type (reviewed in Dong, 2004; Pieterse and Van Loon, 2004). To determine if SA plays a similar role during ARR, the expression of *PR1*, a SA signaling marker, was monitored in young and mature plants. *PR1* expression is reduced in mature compared to young plants after inoculation with *Pst* (Kus et al., 2002; Rusterucci et al., 2005). When this observation is combined with data that illustrates that

NPR1 is not required for ARR it suggests that SA does not play a signaling role during ARR (Kus et al., 2002; Rusterucci et al., 2005).

A number of experiments were conducted to determine if SA plays a role in the intercellular space of plants exhibiting ARR. SA levels were found to rise in intercellular washing fluids (IWFs) and in leaf tissue (after removal of IWFs) of mature plants during ARR (Cameron and Zaton, 2004). IWFs collected from leaves of mature Arabidopsis have elevated levels of SA compared to leaves of young plants after inoculation with *Pst* (Cameron and Zaton, 2004), suggesting that intercellular SA accumulation is important for ARR. Furthermore, IWFs of plants exhibiting ARR display anti-microbial activity, whereas IWFs collected from young plants do not (Kus et al., 2002). SA possesses *in vitro* anti-microbial properties (Sikkema et al., 1995; Johnson et al., 2000; Choi and Gu, 2001; Cameron and Zaton, 2004; Lee et al., 2005), suggesting that it may contribute to the anti-microbial activity in the intercellular space of plants exhibiting ARR.

If intercellular SA is important for ARR, then infiltrating SA into the intercellular space of ARR mutants may rescue them. To test this idea, SA was infiltrated into the leaves of *sid1*, *sid2*, *pad4*, *eds1*, and *iap1-1* (Cameron and Zaton, 2004; Carviel et al., 2009). ARR is partially restored in these plants. However, this partial rescue occurs when leaves of mature plants are challenged with *Pst* at or before 5 hrs post infiltration with SA, but not after 24 hrs post infiltration with SA (Cameron and Zaton, 2004; Carviel et al., 2009). Rescue of the ARR defect of mature *sid2* is associated with elevated SA levels in its intercellular space at 5 hrs post infiltration with SA, whereas little SA is detected in its intercellular space after 24 hrs post infiltration with SA. This suggests that SA must be

present at high levels in the intercellular space to rescue this ARR defect (Cameron and Zaton, 2004). To further test the importance of SA in the intercellular space, the SA hydrolyzing enzyme Salicylate Hydroxylase (SAH) was injected into leaves of mature plants at different time points after inoculation with *Pst* in an attempt to reduce intercellular SA levels (Cameron and Zaton, 2004). This results in a modest decrease in ARR when leaves are injected with SAH up to 24 hpi with *Pst*, however, no decrease in ARR was observed when SAH was injected into leaves at 48 hpi. The SA quantification, addition, and subtraction experiments presented thus far suggest that SA plays an anti-microbial role in the intercellular space during the early stages of ARR (within 24 hpi).

To identify additional genes that play a role during ARR, a reverse genetics approach was utilized. Microarray technology was employed to compare gene expression in leaves collected from mature Arabidopsis plants after inoculation with *Pst* versus plants that were mock-inoculated (Carviel et al., 2009). This experiment identified approximately 100 genes that were up-regulated and 100 genes that were down-regulated during ARR (discussed in Chapter 2). Two *No Apical Meristem Cup-shaped Cotyledon* (*NAC*) transcription factors, *ANAC055* and *ANAC092*, were up-regulated in this microarray.

1.5 The *NAC* gene family

The *NAC* gene family encodes for one of the largest plant-specific transcription factor families (Olsen et al., 2005). It consists of 105 putative members in Arabidopsis (Ooka et al., 2003). NAC proteins are characterized by a conserved, highly charged, N-terminal DNA-binding domain (NAC domain), and a variable transactivation domain at

their C-terminus (Souer et al., 1996; Xie et al., 2000; Duval et al., 2002; Hegedus et al., 2003; Taoka et al., 2004). The NAC domain consists of five conserved motifs (A-E), and it contains putative nuclear localization signals (Xie et al., 2000; Vroeman et al., 2003; Fujita et al., 2004; Tran et al., 2004; Selth et al., 2005). Motifs D and E are necessary for DNA binding (Duval et al., 2002), and motif E is also required for stable dimer formation (Xie et al., 2000).

Genetic and gene expression data suggests that NACs are involved in a variety of plant development processes, including pattern formation in embryogenesis (Souer et al., 1996; Aida et al., 1997; Aida et al., 1999; Takada et al., 2001; Vroemen et al., 2003; Zimmermann and Werr, 2005; Hibara et al., 2006; Smyczynski et al., 2006), seed development (Oh et al., 2005; Uauy et al., 2006), flower development (Souer et al., 1996; Sablowski and Meyerowitz, 1998; Takada et al., 2001; Vroemen et al., 2003; Mitsuda et al., 2005; Hibara et al., 2006), fruit development (Oh et al., 2005; Uauy et al., 2006), leaf development (Hibara et al., 2006; Nikovics et al., 2006), secondary wall synthesis (Zhong et al., 2006), auxin-dependent lateral root formation (Xie et al., 2000), and senescence (John et al., 1997; Guo and Gan, 2006). Furthermore, transcripts of a *NAC* are present in the phloem of pumpkin plants, implicating this NAC in long distance signaling (Ruiz-Medrano et al., 1999).

*NAC*s are expressed in response to a number of biotic and abiotic stresses, including viral infection (*Tomato Leaf Curl Virus* and tomato, Selth et al., 2005; *Pepper Mild Mottle Virus* and pepper, Oh et al., 2005), fungal infection (*Phytophthora infestans* and potato, Collinge and Boller, 2001; *Sclerotinia sclerotiorum* and *Brassica napus*,

Hegedus et al., 2003), bacterial infection (*Pst* and tomato, Mysore et al., 2002; *Xanthomonas campestris* and pepper Oh et al., 2005), insect infestation (flee beetle and *B. napus*, Hegedus et al., 2003), wounding (potato, Collinge and Boller, 2001; pepper Oh et al., 2005; rice, Ohnishi et al., 2005), drought (rice, Ohnishi, 2005; wheat Xue et al., 2006), salt treatment (rice, Ohnishi et al., 2005), and cold conditions (*B. napus*, Hegedus et al., 2003; rice, Ohnishi et al., 2005; wheat, Xue et al., 2006).

NACs are categorized as transcription factors because they activate transcription of a number of reporter genes in yeast (Ren et al., 2000; Xie et al., 2000; Duval et al., 2002; Hegedus et al., 2003; Fujita et al., 2004; Tran et al., 2004; Selth et al., 2005; Hu et al., 2006; Zhong et al., 2006). Also, some NACs bind to a specific DNA sequence in the *Cauliflower Mosaic Virus* 35S (CaMV35S) promoter (AGGATG) (Xie et al., 2000; Duval et al., 2002; Xue et al., 2006), and others bind to a NAC recognition sequence (NACRS: ACACGCATGT) present in promoters of water deficit-inducible genes (Tran et al., 2004; Hu et al., 2006). In addition, some NACs have been observed to localize to the nucleus (Ruiz-Medrano et al., 1999; Xie et al., 2000; Fujita et al., 2004; Taoka et al., 2004; Oh et al., 2005; Selth et al., 2005; Hu et al., 2006; Smyczynski et al., 2006). Interestingly, one NAC (At4g01540) has a transmembrane domain that restricts its localization to the plasma membrane. When its transmembrane domain is cleaved, this NAC is translocated to the nucleus where it up-regulates expression of its regulon (Kim et al., 2006).

There is significant functional redundancy between the NACs. This is exemplified by the Arabidopsis Cup-shaped Cotyledons (CUCs). Single knock-out mutants of *CUC1,*

CUC2, or *CUC3*, exhibit almost no visible phenotype, whereas double mutants display developmental abnormalities (Aida et al., 1997; Aida et al., 1999; Vroemen et al., 2003). However, in some cases inactivating one *NAC* is sufficient to yield a mutant phenotype (Kim et al., 2006; Zhong et al., 2006).

Many studies have characterized the function of NACs by over-expressing them in plants. This alters a number of plant development and stress response processes, including the cell cycle (Kim et al., 2006), cell wall biosynthesis (Zhong et al., 2006), formation of adventitious shoots (Takada et al., 2001), shoot apical meristem formation (Hibara et al., 2003), auxin-responsive pathways (Xie et al., 2000), flowering time (Hegedus et al., 2003; Tran et al., 2004), and water deficit tolerance (Fujita et al., 2004; Tran et al., 2004; Hu et al., 2006; Tran et al., 2007).

Transcriptional regulation of the *NAC* gene family is not very well understood. However, some *NAC*s are expressed in response to treatment with some phytohormones, including SA, JA, MeJA, ET, and ABA (Alonso et al., 2003; Oh et al., 2005; Ohnishi et al., 2005; Xue et al., 2006). Many members of the *NAC* family are regulated at the post-transcriptional level by the microRNA *miR164* (Laufs et al., 2004; Mallory et al., 2004; Guo et al., 2005; Nikovics et al., 2006). *miR164* targets a 21 nucleotide sequence in the activation domain of *NAC* transcripts (Mallory et al., 2004). Over-expressing *miR164*, mutating *miR164*, or engineering *NAC* transcripts to be resistant to *miR164* cleavage results in a wide array of developmental and morphological abnormalities (Laufs et al., 2004; Mallory et al., 2004; Guo et al., 2005; Nikovics et al., 2006). Collectively, this data

illustrates that members of the *NAC* gene family encode for regulators of important processes in plant development and responses to biotic and abiotic stresses.

1.6 Flowering pathways

ARR in Arabidopsis is observed earlier in plants that exhibit accelerated flowering and later in plants delayed in flowering (Leisner et al., 1993; Cecchini et al., 2002; Rusterucci et al., 2005; Goss and Bergelson, 2006). This illustrates an association between ARR and flowering time.

There are four main pathways that regulate flowering time in Arabidopsis (Fig 1.2). Flowering is initiated via the photoperiod or long day pathway when plants are grown under long day conditions or when they are exposed to a few long days. The vernalization pathway initiates flowering in response to exposure to cold temperatures. The gibberellin (gibberellic acid: GA) pathway initiates flowering under short day conditions, and the autonomous pathway initiates flowering under short and long day conditions (Koorneef et al., 1998; Lee et al., 2006; Levy and Dean, 1998; Simpson and Dean, 2002).

Flowering time genes are placed into the different flowering time pathways based on the flowering phenotypes of their mutants. For example, mutants that exhibit altered flowering time only under long days and do not respond to a change in photoperiod, such as the late flowering *constans* (*co*; Putterill et al., 1995), are placed in the photoperiod or long day pathway. Mutants that have altered flowering under both long and short days but still respond to photoperiod by flowering earlier under long day compared to short

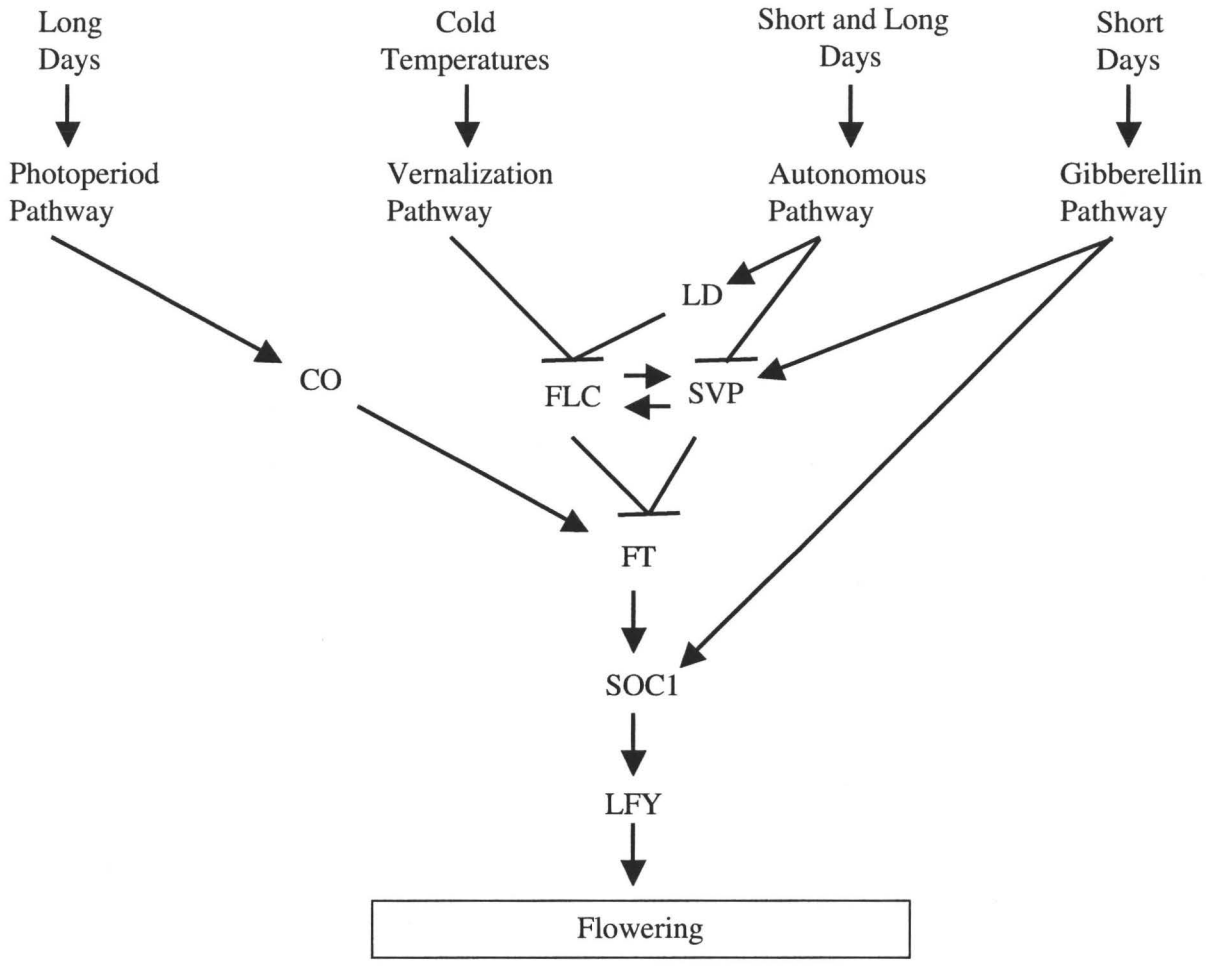


Figure 1.2. Diagram of the main flowering pathways in Arabidopsis. The photoperiod and autonomous pathways promote flowering under long days, the autonomous and gibberellin pathways promote flowering under short days, and the vernalization pathway promotes flowering after exposure to low temperatures. See text for details. Modified from Corbesier and Coupland (2006).

day conditions belong to the autonomous pathway. For example, *luminidependens* (*ld*) flowers late and *short vegetative period* (*svp*) flowers early under short and long days, and both *ld* and *svp* respond to changes in photoperiod (Lee et al., 1994; Aukerman et al., 1999; Hartmann et al., 2000). Therefore, LD and SVP are part of the autonomous pathway. Flowering genes that are expressed in response to GA, such as *SVP* (Li et al., 2008), encode for components of the GA pathway (Blazquez et al., 1997; Eriksson et al., 2006). Therefore, SVP plays a role in the autonomous and GA pathways. Plants that exhibit altered flowering time after vernalization, such as the late flowering *protein arginine methyltransferase5* (*atprm5*: Schmitz et al., 2008), have a compromised vernalization pathway.

The autonomous and vernalization pathways converge on the flowering repressor Flowering Locus C (FLC) (Michaels and Amasino, 1999; Sheldon et al., 2000). Altered flowering of some of the autonomous and vernalization pathway mutants is associated with altered expression of *FLC*. The late flowering *ld* plants, for example, have elevated expression of *FLC* (Aukerman et al., 1999; Domagalska et al., 2007). SVP interacts with FLC and is also a negative regulator of expression of some common target genes such as *Flowering Locus T* (*FT*) and *Suppressor of Overexpression of Constans 1* (*SOC1*) (Li et al., 2008). A number of downstream components are common to most flowering pathways. For example, Leafy (LFY) is a transcription factor that plays a role in all four flowering pathways (Lee et al., 2006).

1.7 Objectives of this thesis

The major goal of this thesis is to increase our understanding of the pathway(s) that contribute to ARR in the Arabidopsis-*Pst* system. More specifically, it explores the role of two NACs, ANAC055 and ANAC092, during ARR. Genetics, molecular biology, and biochemical techniques were employed to identify components of the NAC pathway. The aim of this research is to accomplish three main objectives:

1) Characterize components downstream of the NACs during ARR
2) Identify how expression of the *NAC*s is regulated during ARR
3) Investigate the relationship between flowering and ARR

1.8 Structure of this thesis

This thesis includes four chapters of research (Chapter 2 to Chapter 5) and a concluding chapter (Chapter 6). Chapter 2 introduces the reader to *ANAC055* and *ANAC092* as well as other genes that were identified in an ARR microarray experiment. In Chapter 3, the role of the NACs in JA/ET signaling during ARR is explored. Chapter 4 investigates how JA and SA signaling regulate expression of the *NAC*s. Chapter 5 includes phytohormone analysis (JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA) of plants exhibiting ARR and ARR-incompetent plants, along with *NAC* mutant plants that display ARR defects. A summary of the data is provided in Chapter 6 along with a discussion of the potential role of the NACs during ARR and possible mechanisms that may contribute to ARR. A list of abbreviations used in this thesis is provided, and Appendix A includes all the experiments that were performed.

1.9 Publications arising from this thesis

The data presented in this thesis was or is in the process of being published. Some data presented in Chapter 2 were published in Carviel et al. (2009). Data from Chapter 3, 4, and 5 is being prepared for publication.

Chapter 2:

A microarray experiment identifies a number of genes required for ARR

2.1 Preface

Some of the data presented in Chapter 2 was published in Carviel et al. (2009). The microarray experiment was conducted by Dr. M. Neumann, Dr. N.J. Provart, and Dr. R.K. Cameron (University of Toronto). The data was statistically analyzed by Dr. M. Neumann (University of Toronto), and it was compiled into a table, described, and discussed by F. Al-Daoud and Dr. R.K. Cameron (McMaster University). T-DNA insertion mutants were screened for homozygous plants by Dr. A. Mohammad (McMaster University). Age-related resistance experiments were performed by F. Al-Daoud and Dr. A. Mohammad (McMaster University). Search of the canola EST database was carried out by Dr. M. Champigny (Plant Biotechnology Institute). RT-PCR and in silico promoter analyses was carried out by F. Al-Daoud (McMaster University).

2.2 Abstract

As Arabidopsis (*Arabidopsis thaliana*) ages it exhibits age-related resistance (ARR) to *Pseudomonas syringae* pv. *tomato* (*Pst*). ARR is associated with intercellular salicylic acid (SA) accumulation and the transition to flowering. A microarray was conducted to identify differentially expressed genes during ARR by comparing gene expression between mock-inoculated mature Arabidopsis and plants that were inoculated with *Pst*. This experiment identified more than 100 genes that were up-regulated and more than 100 genes that were down-regulated during ARR. T-DNA insertion lines were obtained for a number of these genes, including two *No Apical Meristem Cup-shaped Cotyledon* (*NAC*) transcription factors, *ANAC055* (At3g15500) and *ANAC092* (At5g39610), a *UDP-glucose Glucosyltransferase*, *UGT85A1* (At1g22400) and a

Cytidine Deaminase, *CDA1* (At2g19570). *anac055*, *anac092*, *ugt85a1*, and *cda1* mutants were compromised in ARR to *Pst*, suggesting that the products of these genes are required for ARR. *In silico* analyses identified putative RAV1 (a member of the APETELLA/Ethylene-Responsive Factor: AP2/ERF transcription factor family) binding sites in the promoter regions of *ANAC055*, *ANAC092*, *UGT85A1*, and *CDA1*. This suggests that these genes may be co-regulated by jasmonic acid/ethylene (JA/ET) signaling during ARR. Lastly, *ANAC055* and *ANAC092* were differentially expressed during ARR in mature plants compared to young plants after inoculation with *Pst*.

2.3 Introduction

Plant pathogen research often focuses on studying plant-microbe interactions at a certain stage of plant development. However, plant resistance to infection by pathogens has been observed to vary as plants age. Young Arabidopsis (*Arabidopsis thaliana*) plants initiate a resistance response known as basal resistance or PAMP-triggered immunity (PTI) when they are infected with the virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 strain. PTI is one of the first lines of pathogen defense triggered by perception of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) by pattern recognition receptors (PRRs) of Arabidopsis (Boller and He, 2009). To counter PTI, *Pst* evolved a number of mechanisms that inhibit plant defenses. For example, it utilizes phytotoxins, such as coronatine, that are secreted in the plant's intercellular space, and protein effectors that are delivered via its type III secretion system (TTSS: Boller and He, 2009).

Resistance to some pathogens increases as some plants mature, and resistance to other pathogens has been observed to decrease with plant age (Panter and Jones, 2002; Whalen, 2005; Develey-Riviere and Galiana, 2007). Increased resistance to pathogens that occurs with plant age is known as age-related resistance (ARR). As Arabidopsis matures it becomes more resistant to virulent and avirulent *Pseudomonas syringae* pv. *tomato* (*Pst*: Kus et al., 2002; Rusterucci et al., 2005), *Ps* pv. *maculicola* (Kus et al., 2002), *P. viridiflava* (*Pv*: Goss and Bergelson, 2006), the oomycete *Hyaloperonospora arabidopsidis* (formerly known as *Hyaloperonospora parasitica* and *Peronospora parasitica*: McDowell et al., 2005; Rusterucci et al., 2005), and Cauliflower Mosaic Virus (CaMV: Leisner et al., 1993). ARR to *Pst*, *Pv*, and CaMV is associated with flowering (Leisner et al., 1993; Cecchini et al., 2002; Kus et al., 2002: Rusterucci et al., 2005; Goss and Bergelson, 2006), and SA is required for ARR to *Pst* and some isolates of *H. arabidopsidis* (Kus et al., 2002; Cameron and Zaton 2004; McDowell et al., 2005; Rusterucci et al., 2005).

In the Arabidopsis-virulent *Pst* (herein referred to as *Pst*) system, there is a decrease in *Pst* levels in mature (up to 100-fold at 5-6 wpg) compared to young plants (3 wpg; Kus et al., 2002). ARR is a whole plant phenomenon since young and mature leaves become more resistant to *Pst* in mature compared to young plants (Kus et al., 2002). ARR is distinct from a number of defense pathways including Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR) since the SAR-defective *non expresser of PR genes1* (*npr1*) and the ISR-defective *ethylene receptor1* (*etr1*) mutant plants exhibit ARR (Kus et al., 2002). The camalexin accumulation-deficient mutant *phytoalexin*

accumulation deficient3 (*pad3*) also exhibits ARR (Kus et al., 2002), suggesting that camalexin is not required for ARR. The *enhanced disease susceptibility7* (*eds7*) mutant impaired in defense to *Pst* and *Psm* also exhibits ARR (Kus et al., 2002). A number of SA accumulation-deficient mutant plants do not exhibit ARR, including *NahG*, *SA induction deficient1* (*sid1*), *sid2*, *pad4*, *eds1*, and *important for ARR pathway1* (*iap1-1*) (Kus et al., 2002; Cameron and Zaton, 2004; Carviel et al., 2009). *NahG* plants express a salicylate hydroxylase protein that converts SA into inert catechol (Gaffney et al., 1993; Vernooij et al., 1995), and *sid1* and *sid2* plants contain mutations in a putative SA transporter, *EDS5* (Nawrath et al., 2002), and an SA biosynthesis gene, *Isochorismate Synthase1* (*ICS1*) (Wildermuth et al., 2001), respectively. Similar to *NahG*, *pad4* accumulates little SA and camalexin in response to *Pseudomonas* infection (Zhou et al., 1998). Taken together, this data suggests that SA is necessary for ARR.

SA usually acts as a signal during resistance to pathogens to up-regulate expression of *PR* genes through activation of NPR1 (Cao et al., 1994; Delaney et al., 1994; Glazebrook et al., 1996; Kinkema et al., 2000; Mou et al., 2003; Shah et al., 1997; Zhang et al., 1999). However, less *PR1* gene expression is observed during ARR in mature plants compared to young plants after inoculation with *Pst* (Kus et al., 2002; Rusterucci et al., 2005). This observation combined with data that showed that *npr1* plants exhibit ARR suggest that SA may not play its traditional signaling role of up-regulating *PR* gene expression during ARR (Kus et al., 2002; Rusterucci et al., 2005). To determine the role of SA during ARR, a number of SA detection, subtraction, and addition experiments were performed. The data suggested that SA plays an anti-microbial

role within 24 hpi in the intercellular space of plants exhibiting ARR (Kus et al., 2002; Cameron and Zaton, 2004; Carviel et al., 2009). Here, a microarray/reverse genetics approach was employed to identify genes required for ARR to *Pst*.

2.4 Results

2.4a Identification of ARR-associated genes using microarray analysis

A microarray experiment was performed to identify genes that are expressed during ARR. Gene expression was compared between mature Col-0 plants (6 wpg) that were mock-inoculated (10 mM $MgCl_2$) or inoculated with *Pst*. It was possible to analyze only one time point after inoculation, therefore samples were collected at 12 hpi based on previous intercellular SA reduction experiments using salicylate hydroxylase that suggest that intercellular SA negatively affects *Pst* growth during ARR between 5 and 24 hours after inoculation (Cameron and Zaton, 2004). RNA extraction and cDNA synthesis was performed on three replicates of each treatment, and each replicate was analyzed using an Affymetrix GeneChip microarray. The signal strength of each gene was averaged over the three replicates of each treatment, and the average signal strength of each gene was compared between samples that were mock-inoculated or inoculated with *Pst*. Significance Analysis of Microarrays (SAM; Tusher et al., 2001) determined which genes were significantly up- or down-regulated. SAM identifies fewer false positives than an average t-test when dealing with microarray data (Tusher et al., 2001). A delta value of 1.483 resulted in the identification of 231 differentially expressed genes with a corresponding false discovery rate (q-value) of < 15%. SAM identified 125 significantly up-regulated and 105 significantly down-regulated genes. The complete list of genes

identified in this microarray is available at http://bar.utoronto.ca/affydb/cgi-bin/affy_db_proj_browser.cgi. Genes up-regulated greater than 2-fold are listed in Table 2.1a and some significantly down-regulated genes are listed in Table 2.1b.

Three members of the *Cytochrome P450* (*CYP*) gene family were differentially expressed in the microarray. The gene product of the most highly up-regulated microarray gene, *CYP71A13* (~3.5-fold up-regulation: Table 2.1a), plays a role in camalexin production in Arabidopsis (Nafisi et al., 2007). *CYP85A2* was also up-regulated (~1.4-fold), and it encodes a protein that is involved in brassinosteroid biosynthesis, which is important for leaf and flower development, flowering time, and defense to pathogens (Nakashita et al., 2003; Kim et al., 2005; Nomura et al., 2005). Little is known about the biochemical role of the third CYP, CYP72A15 (~0.77-fold). CYPs are associated with a number of abiotic and biotic stress responses, and in the production of secondary metabolites, such as phenylpropanoids, jasmonic acid (JA), gibberellic acid (GA), and camalexin (Schuler and Werck-Reichhart, 2003; Ehlting et al., 2006). These CYPs may play a role in secondary metabolites biosynthesis during ARR.

Table 2.1a. Microarray of ARR-associated genes (up-regulated ≥ 2-fold)[a]

AGI#[b]	Probe ID # (affynames)[c]	Transcript Signal Ratio[d]	q value (%)[e]	Functional annotation[f]
At2g30770	267567_at	3.5	3.8	CYP71A13: cytochrome P450 71A13 may be involved in defense response, camalexin biosynthesis, indoleacetaldoxime dehydratase activity.
At5g24780 At5g24770	245928_s_at	2.8	3.0	VSP1 and VSP2: vegetative storage protein 1, putative phosphatase activity, defense,jasmonic acid responses. VSP2 putative phosphatase activity, anti-insect activity, induced by abscisic acid, jasmonic acid, salt, water deficiency, senescence, insect feeding, and wounding.
At1g75750	262947_at	2.7	3.0	GASA1: GA-responsive GAST1 may be responsive to gibberellic acid, abscisic acid, brassinosteriods, may be localized to the cell wall
At3g47420	252414_at	2.4	3.0	Glycerol-3-phosphate transporter, putative cell membrane location, carbohydrate transport,sugar:hydrogen symporter activity.
At3g01970	258975_at	2.3	3.8	WRKY45: putative WRKY transcription factor
At5g39610	249467_at	2.3	3.7	ANAC092: NAC transcription factor
At3g15500	258395_at	2.3	3.5	ANAC055: NAC transcription factor
At5g56870	247954_at	2.2	3.7	ß-GAL4: beta-galactosidase 4, putative carbohydrate metabolism.
At5g13080	245976_at	2.1	3.8	WRKY75: putative WRKY transcription factor involved in nutrient import, lateral root development, response to stress, nutrients levels
At2g19570	265943_at	2.1	3.7	CDA1: cytidine deaminase 1
At1g22400	261934_at	2.1	3.8	UGT85A1: UDP-glucosyl transferase 85A1
At3g50770	252136_at	2.0	3.0	CML41: calmodulin-related protein 41, putative calcium ion binding
At1g70690	260179_at	2.0	3.7	Kinase receptor-like protein, putative function in response to pathogens.

[a]Gene expression in mature plants was compared between mock- and *Pst*-inoculated plants at 12 hpi.
[b]Gene AGI number
[c]Probe ID number
[d]Transcript Signal Ratio represents the fold change in transcript signal after *Pst*-inoculation compared with mock-inoculation
[e]q value represents the false discovery rate assigned by SAM
[f]Functional annotation according to The *Arabidopsis* Information Resource 8 (TAIR 8), May 16, 2008

Table 2.1b. Most down-regulated genes in the ARR Microarray [a]

AGI# [b]	Probe ID # (affynames) [c]	Transcript Signal Ratio [d]	q value (%) [e]	Functional annotation [f]
At2g47880	266516_at	0.31	3.4	GRXC13: glutaredoxin 13. Arsenate reductase protein may have thiol-disulfide exchange intermediate activity involved in electron transport.
At5g62570	247426_at	0.44	3.8	Calmodulin binding protein may be expressed in flowers, roots, And stems.
At4g34950	253215_at	0.52	3.8	Nodulin family protein is a member of the major facilitator superfamily.
At5g49630	248619_at	0.57	3.8	AAP6: amino acid permease and transporter may be located in membrane, down-regulated by water and salt, and expressed in xylem.
At4g17140	245433_at	0.57	3.8	Pleckstrin homology (PH) domain-containing protein. Similar to C2 domain-containing protein.
At4g18250	254660_at	0.58	3.8	Transmembrane receptor serine/threonine kinase-like protein.
At2g29120	266782_at	0.59	3.8	ATGLR2.7: glutamate receptor 2.7. Ligand-gated ion channel Protein may be involved in cellular calcium ion homeostasis. It may be localized to the membrane and endomembrane system.
At4g32060	253457_at	0.60	3.8	Calcium ion-binding EF hand family protein.
At2g32680	267546_at	0.60	3.8	Kinase protein that may contain leucine-rich repeats (LRR). It may be involved in disease resistance, and it may be localized to the enodmembrane system.
At5g38210	249550_at	0.61	3.8	Serine/threonine kinase-like protein may be localized to the endomembrane system.
At2g36630	265203_at	0.62	3.7	Unknown protein that may contain an N-terminal myristoylation domain, and it may be localized to the membrane and endomembrane sytem.
At2g16480	263605_at	0.62	3.8	SWIB complex BAF60b domain-containing protein. Similar to Zinc finger family protein. It may be localized to the nucleus andendomembrane system.
At2g39340	267015_at	0.63	3.8	SAC3/GANP protein family member. Similar to Nucleic acid binding protein.
At5g64080	247268_at	0.63	3.8	Lipid transfer protein (LTP) may be membrane-associated.
AtCg00180	244998_at	0.64	3.0	RPOC1: DNA-directed RNA polymerase may be located in chloroplast.
At5g18470	249983_at	0.64	3.8	Curculin-like lectin protein kinase protein family member. Sugar-binding protein may be located in cell wall.

[a]Gene expression in mature plants was compared between mock- and *Pst*-inoculated plants at 12 hpi.
[b]Gene AGI number
[c]Probe ID number
[d]Transcript Signal Ratio represents the fold change in transcript signal after *Pst*-inoculation compared with mock-inoculation
[e]q value represents the false discovery rate assigned by SAM
[f]Functional annotation according to The Arabidopsis Information Resource 8 (TAIR 8) as of May 16, 2008

Members of the *NAC* (petunia *No Apical Meristem* and Arabidopsis *ATAF1*, and *Cup-shaped Cotyledons*) and *WRKY* plant transcription factor families were up-regulated in the microarray (*ANAC055* and *ANAC092* ~2.3-fold, *ANAC029* ~1.8-fold, *WRKY45* ~2.3-fold, *WRKY75* ~2.1-fold: Table 2.1a). The NAC and WRKY families are implicated in a number of biotic and abiotic stress responses (Rushton and Somssich, 1998; Olsen et al., 2005). *ANAC055* and *ANAC092* are expressed in response to NaCl and abscisic acid (ABA) treatments (Tran et al., 2004; He et al., 2005). In addition, expression of *ANAC055* is up-regulated in response to methyl jasmonate (MeJA) treatment (He et al., 2005; Bu et al., 2008). ANAC029 appears to be a positive regulator of the onset of leaf senescence (Guo and Gan, 2006). NAC and WRKY proteins may contribute to ARR gene regulation.

A *Cytidine Deaminase* was also up-regulated in the microarray (*CDA1* ~2.1-fold; Table 2.1a). CDAs play a role in RNA editing by converting cytidine to uridine (Faivre-Nitschke et al., 1999; Vincenzetti et al., 1999; Gott and Emeson, 2000). Therefore, post-transcriptional gene regulation may be important during ARR.

Two *Uridine Diphosphate-Glucosyltransferase* (*UGT*) genes were identified in the microarray. *UGT85A1* was up-regulated (~2.1-fold: Table 2.1a), while *UGT73B2* was down-regulated (~0.8-fold). Expression of *UGT85A1* is observed in leaf, stem, flower, and root tissue (Woo et al., 2007), whereas *UGT73B* is expressed in response to some pathogens, oxidative stress, wounding and SA or MeJA application (Langlois-Meurinne et al., 2005). Arabidopsis contains ~120 putative UGTs (Li et al., 2001). They are involved in conjugation of glucose to proteins and secondary metabolites, including SA

(Yalpani et al., 1992; Jones et al. 1999, Li et al., 2001). The ARR-associated UGTs may be involved in modifying SA or another metabolite that is important for ARR.

Four *Glutaredoxin* (*GRX*) genes, members of the *Thioredoxin* (*TRX*) superfamily, were differentially expressed in this microarray. *GRXC13* was the most down-regulated gene (~0.3-fold: Table 2.1b). Three other *GRX* genes were up-regulated (At5g18600 ~1.5-fold, At5g63030 ~1.4-fold, and At5g39950). *GRX* genes are expressed in response to oxidative stress and GRX proteins scavenge reactive oxygen species (ROS; Lemaire, 2004). A GRX is thought to participate in modifying the redox state of a TGA transcription factor involved in SAR (Ndamukong et al., 2007). GRXs may be involved in scavenging ROS during ARR and/or altering the redox state of proteins important for ARR.

Genes that encode for proteins that are thought to be involved in Ca^{2+} signaling, such as *CML41* (*Calmodulin-like* (*CML*): ~2.0-fold: Table 2.1a), the Ca^{2+} channel *ATGLR2.7* (*Glutamate Receptor 2.7*: ~0.59-fold), a Ca^{2+}-binding protein (*At4g32060*: ~0.60-fold), and a calmodulin-binding protein (*At5g62570*: ~0.44-fold) were differentially expressed during ARR. This suggests that regulation of cellular Ca^{2+} levels is important for ARR, as is the case for many cellular signaling pathways (McCormack et al., 2005).

A member of the *Lipid Transfer Protein* (*LTP*) gene family was down-regulated in the microarray (At5g64080, ~0.6-fold: Table 2.1b). Some LTPs have been shown to transfer lipids between membranes *in vitro*, while others are associated with wax assembly, and pathogen defense (Garcia-Olmedo et al., 1995; Clark and Bohnert et al.,

1999; Arondel et al., 2000). DIR1, a putative LTP, is involved in systemic signaling during SAR, and accumulation of its transcript is down-regulated in response to pathogen attack (Maldonado et al., 2002). LTPs may play a role in signaling during ARR.

2.4b Microarray/reverse genetics identifies genes required for ARR to *Pst*

By combining the ability to identify ARR-associated genes using microarray analysis with reverse genetics using T-DNA insertion lines (Arabidopsis Biological Resource Center, ABRC), genes that are not only up-regulated during ARR, but whose gene products contribute to ARR can be identified. A number of genes that were up-regulated in the ARR microarray, including *ANAC055* (SALK_014331), *ANAC092* (SALK_090154), *CDA1* (SALK_036597), and *UGT85A1* (SALK_085809), were listed as having T-DNA insertion mutant plants available from ABRC. Plants containing homozygous T-DNA insertions were identified by PCR. RT-PCR with gene-specific primers was performed to confirm that the T-DNA insertion reduced expression of *ANAC055*, *ANAC092*, *CDA1*, and *UGT85A1* in their respective mutant plants (Fig 2.1). RT-PCR analysis demonstrated that both *ANAC092* and *ANAC055* gene expression was abolished in the *anac092* and *anac055* mutants confirming the He et al. (2005) study with *anac092* (Fig 2.1a). However, expression of *UGT85A1* and *CDA1* in the *ugt85A1* and *cda1* mutants was not completely absent, but their expression was reduced compared to the wild-type (Fig 2.1b,c).

The T-DNA mutants were assayed for ARR by comparing *in planta Pst* levels in young and mature plants compared to Col-0 control plants. During ARR experiments

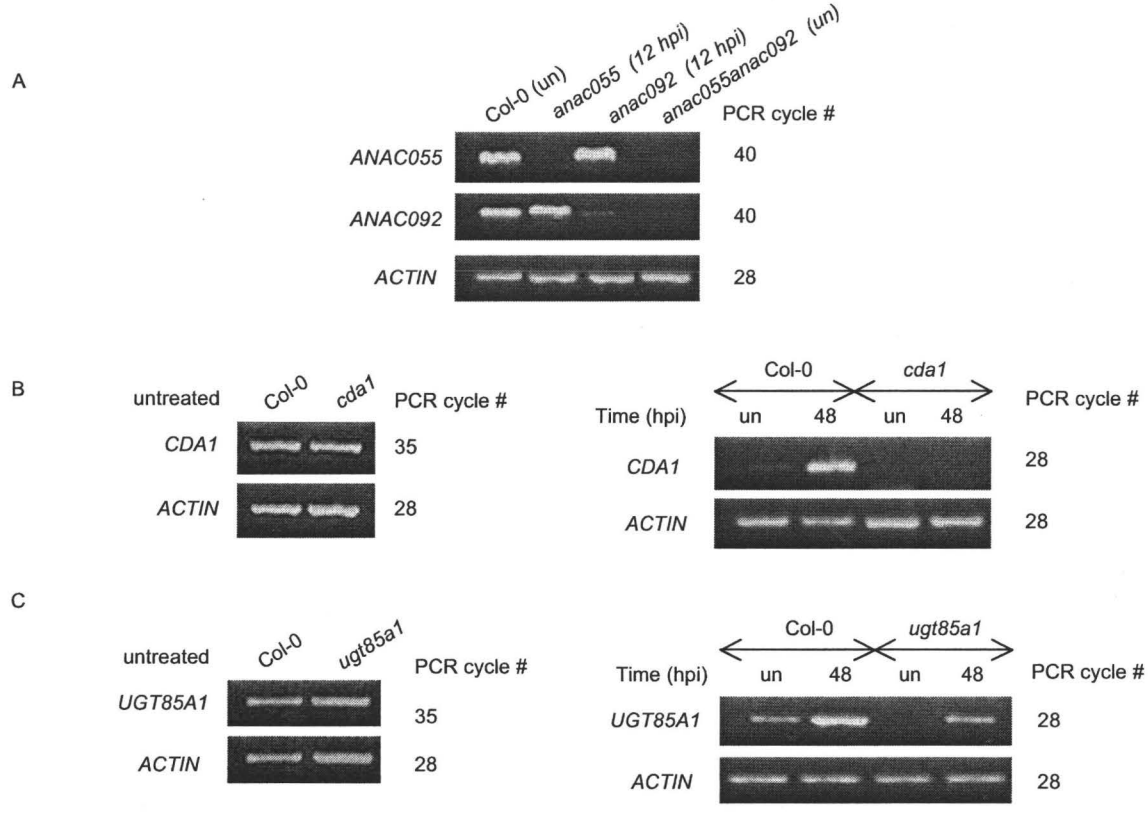


Figure 2.1. ARR gene expression analysis in T-DNA insertion mutant plants. A, RT-PCR with primers for *ANAC055* and *ANAC092* was performed on untreated Col-0 and *anac055anac092* leaf samples and leaves collected from mature (6 wpg) *anac055* and *anac092* plants at 12 hpi with *Pst.* B, RT-PCR with primers for *CDA1* was performed on untreated Col-0 and *cda1* leaf samples (left panel) and leaves collected from untreated (un) mature (6 wpg) Col-0 and *cda1* plants and leaves collected at 48 hpi with *Pst* (right panel). C, RT-PCR with primers for *UGT85A1* was performed on untreated Col-0 and *ugt85a1* leaf samples (left panel) and leaves collected from untreated (un) mature (5 wpg) Col-0 and *ugt85a1* plants and samples collected at 48 hpi with *Pst* (right panel). *ACTIN* was used as a loading control. The number of PCR cycles used is shown.

plants grown under short day conditions (9 hr photoperiod) are inoculated with 10^6 colony-forming units per ml (cfu ml^{-1}) of *Pst*, and *in planta Pst* density is determined three days post inoculation (dpi). Mature, six week-old *anac055*, *anac092*, *cda1* and *ugt85A1* were more susceptible to *Pst* compared to wild-type ARR-competent Col-0 (Fig 2.2). Both *anac092* and *anac055* were compromised in ARR as demonstrated by a modest 10- and 14-fold reduction in *Pst* levels in mature compared to young plants, respectively, compared to a full ARR response characterized by a 154-fold *Pst* reduction in mature compared to young wild-type Col-0 (Expts 1 and 2; Fig 2.2). The *cda1* mutant was compromised in ARR, but to a lesser extent as it still displayed a 65-fold reduction in *Pst* density in mature compared to young plants (Expt 3; Fig 2.2). Even though the ARR response was rather modest in Col-0 in the *ugt85A1* experiment, there was little reduction in *Pst* density in mature compared to young *ugt85A1* plants indicating that *ugt85A1* is compromised in ARR (Expt 4; Fig 2.2).

Another way to quantify ARR is to compare *Pst* levels in mature ARR-competent Col-0 versus each mutant (Expts 1 to 4; Fig 2.2), such that higher *Pst* levels in the mutants equals greater susceptibility or compromised ARR. Compared to mature Col-0, mature *anac055* and *anac092* plants were seven-fold more susceptible, mature *cda1* plants were two-fold more susceptible and mature *ugt85A1* plants were five-fold more susceptible to *Pst* (Student's T-test $P < 0.05$ between each Col-0/mutant pair in Fig 2.2). In at least two other experiments (see legend of Fig 2.2), similar results were observed (*anac055 and anac092* were two- to seven-fold more susceptible, *cda1* was two- to six-

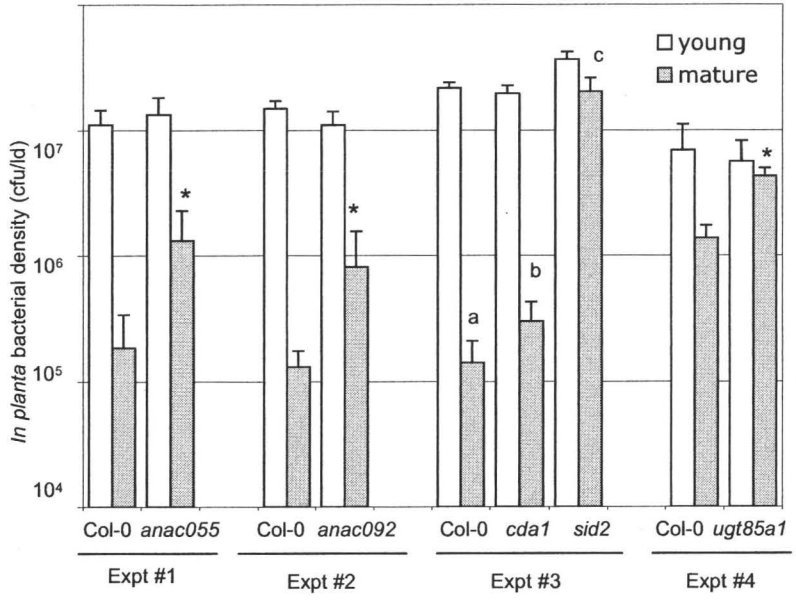


Figure 2.2 *In planta Pst* levels (logarithmic scale) in young or mature Col-0, *anac092*, *anac055*, *cda1*, *ugt85A1* and *sid2*. Plants were inoculated with 10^6 cfu ml^{-1} *Pst* at 3, 5 or 6 wpg. *In planta* bacterial levels were monitored 3 dpi and are presented as the mean ± SD of three samples. Col-0 controls are presented for each independent experiment. Significant differences between mature Col-0 and mutants indicated by *, $P < 0.05$ student's T-Test. Different letters represent significant differences between mature genotypes in Expt. 3 (Tukey's Honest Significant Difference, $P < 0.05$). These experiments were performed at least three times with similar results (see Appendix A Fig A2 and Fig A4 for replicates).

fold more susceptible, and *ugt85A1* was three- to five-fold more susceptible than mature Col-0). These data suggest that *anac055*, *anac092*, *cda1*, and *ugt85A1* mutants are compromised in ARR. However, mature mutant plants still supported a 10- to 65-fold decrease in *Pst* density compared to young mutant plants (Fig 2.2), suggesting that ARR is not completely abolished in these plants.

PTI to *Pst* in young plants was also monitored in these ARR mutants to determine if these ARR gene products are also required for PTI. Young wild-type Col-0 exhibit some residual PTI in spite of the fact that *Pst* negatively regulates PTI. This is illustrated by the fact that some defense mutants, including *sid2* that is compromised in SA biosynthesis, display reduced PTI in young plants (young *sid2* were two-fold more susceptible to *Pst* compared to the wild-type; Student's T-test $P < 0.05$; Expt 3; Fig 2.2). Young *anac055*, *anac092*, *cda1* and *ugt85A1* plants supported high *in planta Pst* levels similar to Col-0 (~10^7 cfu ld^{-1}, Fig 2.2), suggesting that ANAC055, ANAC092, CDA1 and UGT85A1 are not required for PTI.

2.4c *In silico* promoter analysis of *ANAC055*, *ANAC092*, *CDA1*, and *UGT85A1*

ANAC055, *ANAC092*, *CDA1*, and *UGT85A1* were up-regulated in the ARR microarray, suggesting that they may be co-regulated during ARR. If these genes are co-regulated during ARR, then they may possess similar cis-acting elements in their promoter regions. To identify putative cis elements present in promoter regions of these ARR genes, the Arabidopsis Cis-Regulatory Database (AtCisDB) at the Ohio State University Arabidopsis Gene Regulator Information Server (AGRIS: http://arabidopsis.med.ohio-state.edu/AtcisDB/) was used to analyze the promoter region

(an ~3 Kb region upstream of the ATG) of *UGT85A1*, *CDA1*, *ANAC055*, and *ANAC092*. All four genes had at least three putative GATA and RAV1 (also known as Ethylene-Responsive DNA-binding Factor 4: EDF4) binding sites in their promoter regions (Table 2.2; as of October 14, 2009). This suggests that GATA and RAV1 transcription factors play a role in regulating these genes. RAV1 belongs to the APETELLA2/Ethylene-Responsive Factor (AP2/ERF) superfamily (Kagaya et al., 1999) which regulates a number of jasmonic acid and ethylene (JA/ET)-associated defense responses (Gutterson and Reuber, 2004). For example, putative RAV1 binding sites are present in the promoter region of the JA/ET-associated *Plant Defensin1.2* (*PDF1.2*) defense gene (Brown et al., 2003). GATA transcription factors regulate light-responsive genes (Teakle et al., 2002; Manfield et al., 2007). Taken together, this data suggests that *ANAC055*, *ANAC092*, *UGT85A1*, and *CDA1* may be regulated by RAV1 transcription factors in a JA/ET-associated manner during ARR, and they encode for proteins that may play a role in developmental processes such as responses to light. Furthermore, *ANAC055* and *ANAC092* have at least three putative MYC2/JIN1, LFY, and Ibox binding sequences in their promoter regions (Table 2.2c,d). This suggests that *ANAC055* and *ANAC092* are co-regulated by MYC2/JIN1, LFY, and Ibox-binding transcription factors. MYC2/JIN1 regulates JA signaling during defense responses (Dombrecht et al., 2007), the Ibox is present in promoters of light-regulated genes (Giuliano et al., 1988), and LFY is involved in regulating flowering time genes (Corbesier and Coupland, 2006). These data suggest that *ANAC055* and *ANAC092* may be regulated by RAV1 and MYC2/JIN1 during ARR,

Table 2.2a. Putative *cis*-acting elements found in the promoter of *UGT85A1* with AtcisDB

Binding Site Name[a]	Binding Site Genome Start Site[b]	Binding Site Genome End Site[c]	Binding Site Sequence[d]	Transcription Factor Family[e]
AtMYB2	7907063	7907069	ctaacca	MYB
AtMYC2	7909239	7909244	cacatg	BHLH
	7909240	7909245		
	7908504	7908509		
Bellringer/replumless/pennywise	7907822	7907829	aaattaaa	Homeobox
	7908873	7908880		
ATB2/AtbZIP53/AtbZIP44/GBF5	7907264	7907269	actcat	BZIP
W-box	7909060	7909065	ttgact	WRKY
	7908735	7908740		
	7908678	7908683		
ARF1	7906983	7906988	tgtctc	ARF
	7908477	7908482		
	7907001	7907006		
DPBF1&2	7907193	7907199	acacaag	BZIP
	7908064	7908070	acacagg	
	7909476	7909482	acacgtg	
	7908541	7908547	acacgag	
	7907911	7907917		
MYB1	7906930	7906937	atccaacc	MYB
MYB4	7907420	7907426	aacaacc	MYB
	7908991	7908997		
	7909522	7909528		
RAV1-A	7906968	7906972	caaca	ABI3VP1
	7907419	7907423		
	7909311	7909315		
	7907324	7907328		
	7907251	7907255		
	7907182	7907186		
TGA1	7907897	7907904	tgacgtgg	BZIP
LFY	7907657	7907662	ccattg	LFY
	7908520	7908525		
	7907777	7907782		
	7907750	7907755		
	7907657	7907662	ccaatg	
ABRE-like	7907898	7907905	gacgtggc	N/A
ARF	7906983	7906988	tgtctc	
	7908477	7908482		
	7907001	7907006		
BoxII	7907805	7907810	ggttaa	N/A
	7909013	7909018		
GATA [Light-Responsive Element]	7907118	7907123	agataa	N/A
	7909398	7909403		
	7908423	7908428		
	7907205	7907210	tgataa	
	7908600	7908605		
	7906775	7906780		
	7907274	7907279	agatag	
	7909232	7909237		
G-box [Light-Responsive Element]	7909477	7909482	cacgtg	N/A
	7909476	7909481		
Ibox promoter motif	7908579	7908584	gataag	N/A
T-box promoter motif	7907299	7907304	actttg	N/A
	7908481	7908486		
	7907450	7907455		
SORLREP1	7909114	7909123	ttatactagt	N/A
SORLIP1	7907160	7907165	agccac	N/A
SORLIP2	7907854	7907858	gggcc	N/A
SORLIP4	7907395	7907403	gtatgatgg	N/A

[a] Name of binding site according to AtcisDB (as of October 15, 2009)
[b] Start site of the binding site in the *Arabidopsis* genome
[c] End site of the binding site in the *Arabidopsis* genome
[d] Sequence of the binding site found by AtcisDB
[e] Name of the transcription factor family that binds to the *cis*-acting element according to AtcisDB. N/A = not available

Table 2.2b. Putative *cis*-acting elements found in the promoter of *CDA1* with AtcisDB

Binding Site Name[a]	Binding Site Genome Start Site[b]	Binding Site Genome End Site[c]	Binding Site Sequence[d]	Transcription Factor Family[e]
W-box	8479364	8479369	ttgact	WRKY
DPBF1&2	8479126	8479132	acaccgg	BZIP
MYB4	8478892	8478898	aacaaac	MYB
	8478753	8478759		
	8479424	8479430	accaaac	
	8479331	8479337	aactacc	
RAV1-A	8478992	8478996	caaca	ABI3VP1
	8479068	8479072		
	8479124	8479128		
	8478923	8478927		
	8478756	8478760		
BoxII	8478975	8478980	ggttaa	N/A
	8478965	8478970		
GATA [Light-Responsive Element]	8479149	8479154	tgatag	N/A
	8479078	8479083		
	8478987	8478992	tgataa	
	8478851	8478856		
	8478880	8478885		
Ibox	8479033	8479038	gataag	N/A
	8478879	8478884		

[a] Name of binding site according to AtcisDB (as of October 15, 2009)
[b] Start site of the binding site in the *Arabidopsis* genome
[c] End site of the binding site in the *Arabidopsis* genome
[d] Sequence of the binding site found by AtcisDB
[e] Name of the transcription factor family that binds to the *cis*-acting element according to AtcisDB. N/A = not available

Table 2.2c. Putative *cis*-acting elements found in the promoter of *ANAC055* with AtcisDB

Binding Site Name[a]	Binding Site Genome Start Site[b]	Binding Site Genome End Site[c]	Binding Site Sequence[d]	Transcription Factor Family[e]
AtMYC2	5232882	5232887	cacatg	BHLH
	5234040	5234045		
	5233315	5233320		
	5232450	5232455		
ATB2/AtbZIP53/	5234104	5234109	actcat	BZIP
AtbZIP44/GBF5	5234237	5234242		
W-box	5232952	5232957	ttgacc	WRKY
	5231813	5231818		
DPBF1&2	5234033	5234039	acacaag	BZIP
	5233797	5233803		
	5234124	5234130	acacgtg	
	5234510	5234516		
	5234124	5234130		
	5234605	5234611	acacgag	
	5233950	5233956		
	5234040	5234046	acacatg	
	5232450	5232456		
	5233073	5233079	acacagg	
MYB3	5231897	5231904	taactaac	MYB
MYB4	5231898	5231904	aactaac	MYB
	5232365	5232371	aacaaac	
	5233955	5233961		
	5234055	5234061	aactacc	
RAV1-A	5231764	5231768	caaca	ABI3VP1
	5232612	5232616		
	5233096	5233100		
	5234425	5234429		
	5233958	5233962		
	5231757	5231761		
TGA1	5232918	5232925	tgacgtgg	BZIP
LFY	5234097	5234102	ccagtg	LFY
	5234367	5234372	ccattg	
	5232755	5232760	ccaatg	
ABRE-like	5234511	5234518	cacgtgtc	N/A
	5234508	5234515	cacgtgga	
	5234122	5234129	cacgtgta	
	5232917	5232924	gacgtgga	
	5232521	5232528	tacgtgta	
BoxII	5232791	5232796	ggttaa	N/A
	5233666	5233671		
GATA [Light-Responsive	5231964	5231969	agataa	N/A
Element]	5233087	5233092		
	5233056	5233061		
	5233507	5233512	tgataa	
	5234520	5234525		
	5232686	5232691		
	5232196	5232201		
	5232098	5232103		
	5231790	5231795		
	5233412	5233417	tgatag	
G-box [Light-Responsive	5234125	5234130	cacgtg	N/A
Element]	5234511	5234516		
	5234510	5234515		
	5234124	5234129		
GCC-box	5234570	5234575	gccgcc	N/A
Ibox	5231965	5231970	gataag	N/A
	5232135	5232140		
	5231789	5231794		
PI	5233319	5233326	gtgatcac	N/A
	5233318	5233325		
T-box	5231719	5231724	actttg	N/A
	5232765	5232770		
	5233148	5233153		
SORLREP3	5234594	5234602	tgtatatat	N/A

[a] Name of binding site according to AtcisDB (as of October 15, 2009)

[b] Start site of the binding site in the *Arabidopsis* genome

[c] End site of the binding site in the *Arabidopsis* genome

[d] Sequence of the binding site found by AtcisDB

[e] Name of the transcription factor family that binds to the *cis*-acting element according to AtcisDB. N/A = not available

and their gene products may contribute to plant development processes such as flowering time and responses to light.

2.4d ARR gene expression analysis

Since ANAC055, ANAC092, CDA1, and UGT85A1 contribute to ARR in mature plants and do not appear to play a role during defense responses to *Pst* in young plants, their gene expression patterns may differ in young ARR-incompetent plants compared to mature plants exhibiting ARR. RT-PCR was performed on leaves collected from young (*in planta Pst* levels ~10^7 cfu ld^{-1}) and mature (*in planta Pst* levels ~10^5 cfu ld^{-1}) Col-0 plants at < 5 minutes post inoculation (mpi) and at 12, 24, and 48 hpi with *Pst* (Fig 2.3) using primers for *ANAC055*, *ANAC092*, *CDA1*, *UGT85A1*, and *ACTIN1* as an internal loading control (Table 2.3). *CDA1* and *UGT85A1* were similarly expressed in young and mature plants. *ANAC055* was more highly expressed at 48 hpi in young compared to mature plants. Little expression of *ANAC092* was observed in young plants until 48 hpi, however it was expressed to moderate levels throughout ARR in mature plants (5 mpi, 12, 24 and 48 hpi). Furthermore, *ANAC092* was expressed to higher levels at 5 mpi, 12, and 24 hpi in mature plants compared to young plants. Taken together, this data suggests that *ANAC055* is expressed to lower levels during ARR compared to young plants after inoculation with *Pst*, whereas *ANAC092* is expressed earlier during ARR compared to young plants after inoculation with *Pst*.

Table 2.2d. Putative *cis*-acting elements found in the promoter of *ANAC092* with AtcisDB

Binding Site Name[a]	Binding Site Genome Start Site[b]	Binding Site Genome End Site[c]	Binding Site Sequence[d]	Transcription Factor Family[e]
AtMYC2	15878592	15878597	cacatg	BHLH
	15878769	15878774		
	15879020	15879025		
Bellringer/replumless/	15878448	15878455	aaattaaa	Homeobox
Pennywise	15877445	15877452		
ATB2/AtbZIP53/	15877661	15877666	actcat	bZIP
AtbZIP44/GBF5	15877872	15877877		
W-box	15877609	15877614	ttgacc	WRKY
	15879770	15879775		
	15879782	15879787		
	15879436	15879441		
	15877258	15877263	ttgact	
ATHB1	15879775	15879783	caattattg	HB
	15879774	15879782	caataattg	
ATHB2	15878478	15878486	taataatta	HB
ATHB5	15879775	15879783	caattattg	HB
	15879774	15879782	caataattg	
CCA1	15879380	15879387	aacaatct	MYB-related
	15879534	15879541		
	15878437	15878444	aaaaatct	
DPBF1&2	15878634	15878640	acacgtg	bZIP
	15878889	15878895	acactgg	
RAV1-A	15877280	15877284	caaca	ABI3VP1
	15877732	15877736		
	15877810	15877814		
	15877883	15877887		
	15878735	15878739		
	15879060	15879064		
	15878595	15878599		
RAV1-B	15879420	15879425	cacctg	ABI3VP1
LFY	15877581	15877586	ccaatg	LFY
	15877782	15877787	ccattg	
	15878889	15878894	ccagtg	
BoxII	15878150	15878155	ggttaa	N/A
GATA [Light-Responsive	15877238	15877243	agataa	N/A
Element]	15879338	15879343		
	15879679	15879684		
	15877924	15877929	tgataa	
	15878141	15878146		
	15878298	15878303		
	15878305	15878310		
	15879330	15879335		
	15879373	15879378		
	15879386	15879391		
	15879281	15879286		
	15879612	15879617	tgatag	
G-box [Light-Responsive	15878635	15878640	cacgtg	N/A
Element]	15878634	15878639		
GCC-box	15877319	15877324	gccgcc	N/A
Ibox	15878306	15878311	gataag	N/A
	15879374	15879379		
	15879385	15879390		
	15879045	15879050		
L1-box	15879096	15879103	taaatgta	N/A
RY	15878951	15878958	catgcatg	N/A
	15878950	15878957		
T-box	15878710	15878715	actttg	N/A

[a] Name of binding site according to AtcisDB (as of October 15, 2009)
[b] Start site of the binding site in the *Arabidopsis* genome
[c] End site of the binding site in the *Arabidopsis* genome
[d] Sequence of the binding site found by AtcisDB
[e] Name of the transcription factor family that binds to the *cis*-acting element according to AtcisDB. N/A = not available

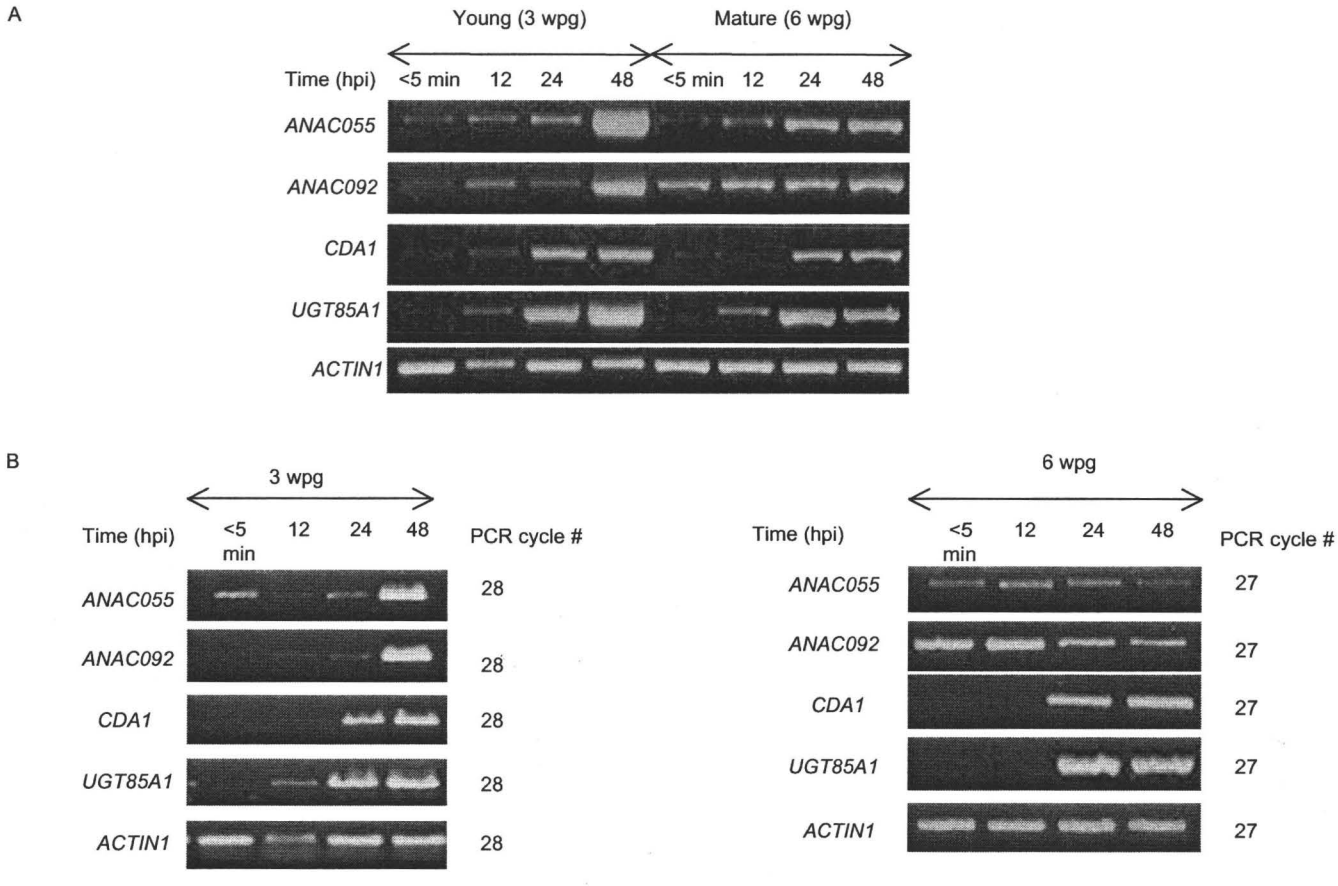


Figure 2.3. ARR gene expression analysis. A, RT-PCR analysis of leaves collected from 3 and 6 wpg Col-0 at <5 min post inoculation (mpi) or 12, 24, and 48 hpi with 10^6 cfu ml^{-1} *Pst*. 28 PCR cycles were used for *ANAC055*, *ANAC092*, *CDA1*, *UGT85A1*, and *ACTIN* primers. B, Replicate of *ANAC055*, *ANAC092*, *CDA1*, and *UGT85A1* gene expression presented in part A.

Table 2.3. Forward and reverse primer sequences used in RT-PCR and cloning

Gene/primer name and AGI number	Primer sequences 5' → 3'
CDA1 At2g19570	F[a] TCCAAAGAAGCAGAATCCGC
	R[b] GTCTCTAATAACAACCTCGCC
UGT85A1 At1g22400	F GTTTGTGTTCCATATCCGGC
	R GAACCATAGCCTCCTCTCC
ANAC055 At3g15500	F ATGGGTCTCCAAGAGCTTGA
	R TCAAATAAACCCGAACCCAC
ANAC092 At5g39610	F ATGGATTACGAGGCATCAAG
	R TCAGAAATTCCAAACGCAAT
ACTIN1 At2g37620	F GGCGATGAAGCTCAATCCAAACG
	R GGTCACGACCAGCAAGATCAAGACG
LBb1[c]	F GCGTGGACCGCTTGCTGCAACT
AttB1NAC3F	F GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGGTCTCCAAGA
AttB2NAC3RNS	R GGGGACCACTTTGTACAAGAAAGCTGGGTGAATAAACCCGAACCC
AttB1NAC5F	F GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGATTACGAGGCATCA
AttB2NAC5R	R GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAGAAATTCCAAACGCAATC
AttB435SF	F GGGGACAACTTTGTATAGAAAAGTTGGGAGCTTGCATGCCTGCAGGTC
AttB135SR	R GGGGACTGCTTTTTTGTACAAACTTGGTCTAGAGTCCCCCGTGTTCT

[a] forward primer sequence
[b] reverse primer sequence
[c] T-DNA left border primer sequence

2.4e *Brassica* species contain putative *ANAC055* and *ANAC092* orthologs

One of the long-term goals of this project is to characterize the function of ARR genes, and to transfer this technology to crop plants and improve their resistance to pathogens. To determine if crops, such as canola, possess *NAC* genes similar to *ANAC055* and *ANAC092*, the NCBI database was searched for canola *NAC* genes. A number of previously characterized *NAC*-like genes were found in *Brassica napus* (Hegedus et al., 2003). To determine if the BnNACs were similar to ANAC092 and/or ANAC055, the deduced protein sequences of the *B. napus NAC* (*BnNAC*) and representatives of all the Arabidopsis NAC subfamilies, according to Ooka et al. (2003), were included in a CLUSTAL W analysis through the San Diego Super Computer (Fig 2.4). Most *B. napus* NACs clustered with the ATAF subfamily, including ANAC102. One *B. napus* protein, BnNAC485, clustered with the NAC3 subfamily, which includes ANAC055. No *B. napus* NAC proteins clustered with the NAM subfamily that includes ANAC092. Therefore, *B. napus* possesses ATAF-like and NAM-like *NAC* genes.

To determine if canola expresses any *ANAC092*-like genes, the *ANAC092* nucleic acid sequence was blasted against a canola EST database. An *ANAC092*-like EST was retrieved (Fig 2.5). This suggests that *Brassica* possesses and expresses at least one *ANAC092*-like gene.

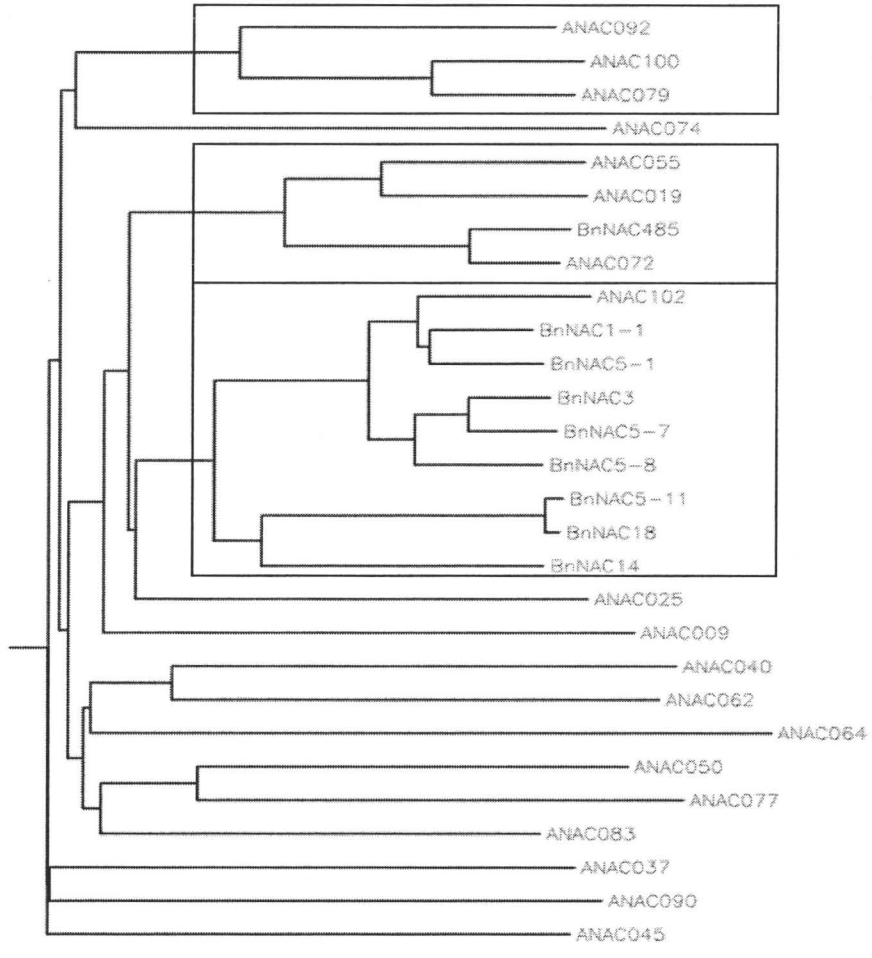


Figure 2.4. Phylogenetic analysis of deduced *Brassica napus* NAC proteins found in the NCBI database with representatives of all the *Arabidopsis* NAC subfamilies according to Ooka et al. (2003). Most of the *B. napus* NACs clustered with the ATAF subfamily, and one clustered with the NAC3 subfamily. No *B. napus* NACs clustered with the NAM subfamily.

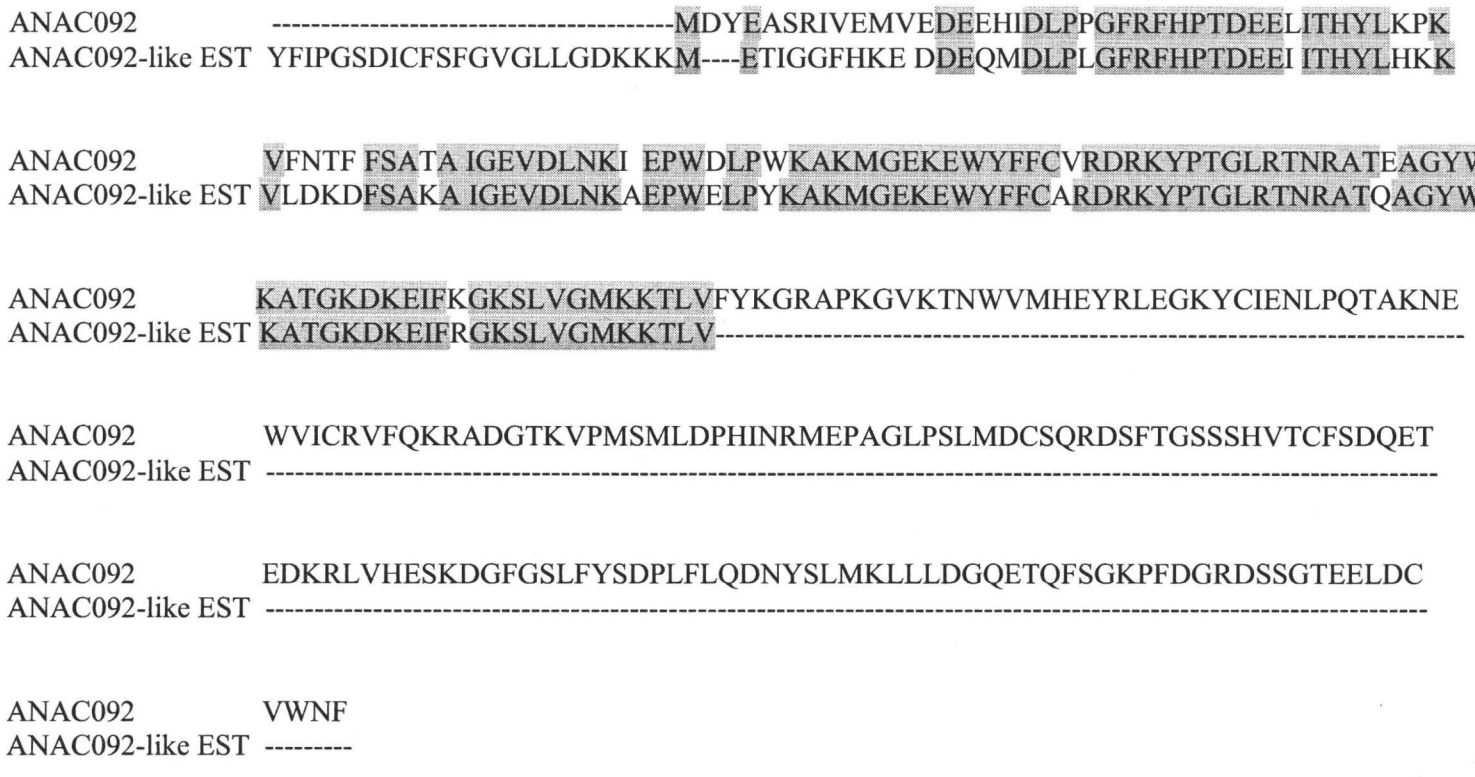


Figure 2.5. Multiple alignment of ANAC092 and *B. napus* ANAC09-like EST deduced amino acid sequences. Common residues are shaded.

2.5 Discussion

2.5a Identification of genes expressed during ARR

ARR microarray analysis at 12 hpi identified over 200 differentially expressed genes. This microarray paints a complex picture of the signaling pathways that may be involved in ARR. The most highly expressed gene in the ARR microarray analysis is *CYP71A13*, which has recently been shown to encode for a protein that is involved in camalexin biosynthesis (Nafisi et al., 2007). However, ARR does occur in the *pad3-1* camalexin biosynthesis mutant (Kus et al., 2002), suggesting that camalexin is not required for ARR. A putative T-DNA insertion mutant in *CYP71A13* is currently being characterized to determine if this CYP450 produces another secondary metabolite that is required during ARR. *CYP85A2* is also up-regulated in the microarray. CYP85A2 is implicated in the brassinosteroid (BR) signaling pathway (Nomura et al., 2005), suggesting that BR signaling may be involved in ARR. BR signaling contributes to resistance to the fungus *Phytophthora infestans* in potato by inducing ethylene production, and it enhances resistance to *Pseudomonas* and Tobacco Mosaic Virus in tobacco in an SA-independent manner (Krishna, 2003).

A gene involved in gibberellic acid (GA) signaling, *GA-responsive-like GA-stimulated Transcript* (*GASA*), and a number of JA-associated genes (*VSP1* and *ANAC055*) are also up-regulated during ARR. This suggested that JA and GA may play a role in ARR. Up-regulation of JA-associated genes during ARR may be due to the *Pst*-produced JA mimic coronatine acting to enhance expression of these genes in an attempt to negatively regulate plant defense (Bender et al., 1999). However, some of these gene

products, including ANAC055, are required for ARR, as demonstrated by the increased susceptibility to *Pst* observed in mature *anac055*. Furthermore, *ANAC055* and *ANAC092* are differentially expressed in mature compared to young plants after inoculation with *Pst*. Since the same *Pst* strain is used to inoculate these plants, and it presumably produces similar amounts of coronatine in plants of different ages, then the different expression patterns of the *NAC*s in young and mature plants may not be entirely due to coronatine. Therefore, expression of *ANAC055* and *ANAC092* during ARR may be partly regulated by the plant and partly regulated by *Pst*.

Taken together, the microarray data suggests that BR, GA, and/or JA may play a signaling role during ARR. This seems likely since SA signaling does not seem to be important for ARR (Kus et al., 2002; Cameron and Zaton, 2004; Rusterucci et al., 2005).

2.5b ANAC055 and ANAC092 may be involved in JA/ET signaling during ARR

Promoter analysis of *ANAC055* and *ANAC092* found putative JIN1/MYC2 and RAV1 (AP2/ERF) binding sites. Also, *ANAC055* is expressed in a *JIN1/MYC2*-dependent manner during fungal infection (Bu et al., 2008). JIN1/MYC2 and AP2/ERF regulate JA/ET signaling during plant defense (Gutterson and Reuber, 2004; Dombrecht et al., 2007), suggesting that they may contribute to JA/ET signaling during ARR. However, the *jin1-1* and *jar1-1* JA signaling mutants are competent for the ARR response (Carviel et al., 2009). This strongly suggests that the JIN1-JAR1 branch of the JA signaling pathway is not required for ARR (Carviel et al., 2009). JIN1/MYC2 may regulate the expression of *ANAC055* and *ANAC092* during some processes, but this does not appear to be required for ARR. Therefore, *ANAC055* and *ANAC092* may be regulated by a *JIN1-*

independent JA/ET pathway during ARR, which may include RAV1 members of the AP2/ERF family.

2.5c ANAC055, ANAC092, CDA1, and UGT85A1 are required for ARR

T-DNA insertion mutants in *ANAC055*, *ANAC092*, *CDA1*, and *UGT85A1* were compromised in ARR to *Pst*, and they have also been shown to be defective in ARR to *H. arabidopsidis* (Carviel et al., 2009). ARR to *Pst*, however, was not completely abolished in these plants suggesting that gene products of other members of these gene families may be compensating for the absence of ANAC055, ANAC092, CDA1, and UGT85A1. Alternatively, there may be other signaling pathways that contribute to ARR that are still active in these mutants.

ANAC055 and ANAC092 belong to the large plant-specific NAC family, consisting of 105 and 75 putative members in Arabidopsis and rice, respectively (Ooka et al., 2003). These transcription factors have a conserved N-terminal DNA-binding (NAC domain) and a variable C-terminal transactivation domain (Souer et al., 1996; Xie et al., 2000; Duval et al., 2002; Hegedus et al., 2003; Taoka et al., 2004). Studies indicate that ANAC055 and ANAC092 regulate expression of genes during abiotic stress responses (Tran et al., 2004; He et al., 2005) and ANAC055 regulates expression of JA-related genes during fungal infection (Bu et al., 2008). Identification of downstream and upstream components of the NAC ARR pathway will provide insights into ARR signaling

2.6 Materials and Methods

2.6a Plant Growth Conditions

The *Arabidopsis thaliana* wild-type Columbia-0 (Col-0) and *sid2* (C. Nawrath, University of Fribourg, Switzerland) were used. Mutants that were examined include *anac055* (SALK_014331), *anac092* (SALK_090154), *cda1* (SALK_036597), *ugt85A1* (SALK_085809) (Arabidopsis Biological Resource Center, Ohio State University, USA, Alonso et al., 2003). Seeds were surface sterilized, germinated and grown as described previously (Rusterucci et al., 2005).

2.6b ARR Assays

Arabidopsis plants were inoculated with virulent *Pseudomonas syringae* pv. *tomato* (*Pst)* strain DC3000 (rifampicin and kanamycin resistant) obtained from Dr. Andrew Bent (University of Wisconsin at Madison) (Whalen et al., 1991). *Pst* was grown to mid-log phase in King's B media and kanamycin (50 $\mu g\ ml^{-1}$) shaken overnight at room temperature (22-25 °C), then diluted to 10^6 colony forming units per ml, (cfu ml^{-1}) in 10 mM $MgCl_2$. Three to four leaves of young plants (3 weeks post germination, wpg) or four to six leaves of mature plants (5-6 weeks post germination, wpg) on seven to twelve plants without visible inflorescence stems were inoculated with virulent *Pst* and *in planta* bacterial levels were determined as previously described (Wolfe et al., 2000; Kus et al., 2002).

2.6c Microarray Analysis

Col-0 plants were grown as described above. Four to five leaves per mature plants (43 dpg) were inoculated with 10 mM sterile $MgCl_2$ (96 in total) and 96 were inoculated

with *Pst*. Three replicate samples containing 0.8-1.0 g of inoculated leaf tissue (~12 leaves) were collected from both treatments at 12 hours post-inoculation (hpi). To ensure that ARR was induced, bacterial growth was monitored using young plants at 21 dpg and mature plants at 46 dpg. Young plants supported a typically high level of *Pst* growth ($1.0 \times 10^7 \pm 1.0 \times 10^6$ cfu ld^{-1}), while mature plants limited *Pst* growth (1.0×10^5 cfu $ld^{-1} \pm 3.6 \times 10^4$). Therefore these mature plants did display ARR.

Total RNA was extracted from each leaf sample replicate (TRIzol, Invitrogen). Ath1 whole genome arrays containing probe sets to investigate the expression of approximately 22800 *A. thaliana* genes were obtained from Affymetrix (Santa Clara, California). Double stranded cDNA was synthesized according to the suppliers instructions from 10 μg of total RNA using the GeneChip T7-Oligo(dT) promoter primer kit (5'GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$3') (Affymetrix) and the SuperScript Choice Kit (Gibco BRL). cDNA was purified using the GeneChip Sample Cleanup Module according to the manufacturer's instructions (Affymetrix) and was used to synthesize biotinylated cRNA using the Enzo BioArray High Yield RNA Transcript Labeling Kit (Affymetrix). Biotinylated cRNA was purified and fragmented according to manufacturer's directions using the GeneChip Sample Cleanup Module (Affymetrix).

Hybridization was performed using the Affymetrix GeneChip fluidics station according to the manufacturer's protocols. The hybridization cocktail for each microarray was prepared using 20 μg of fragmented biotinylated cRNA as well as the recommended eukaryotic hybridization controls. Hybridization was performed at 45 °C for 16 hours.

The microarrays were washed and stained with a streptavidin phycoerythrin conjugate using the Antibody Amplification for Eukaryotic Targets protocol recommended by Affymetrix.

Microarrays were scanned using an Agilent Gene Array Scanner and the images were quantitatively analyzed using the Affymetrix Microarray Suite Version 5.0. Each image was visually inspected for hybridization artifacts and manufacturing defects in the construction of the chip. The signal intensities of the eukaryotic hybridization controls and internal control genes fell within the expected ranges, indicating that RNA sample integrity and hybridization efficiency was high and comparisons could be made both within and between the mock and *Pst* treatments. Experimental variation was minimized by applying a global method of normalization to the microarray data. The scaling/normalization factors applied to each of the six microarrays differed from each other by less than 3-fold indicating that the arrays were comparable and that sample degradation was unlikely to have occurred. Furthermore, the proportion of probe sets called 'present' compared to the complete probe set was similar for all replicates.

The detection algorithm was set to use the default as the threshold for determining whether a measured transcript was considered present, absent, or marginal. Only data from those transcripts which were designated marginal or present in each replicate for each of the two treatments were retained for further analysis.

To identify genes that may be involved in the ARR response, a 2-class SAM (Tusher et al., 2001) was performed to provide a list of genes with average Mock/Virulent signal intensity ratios that were significantly different from 1.0. A delta

value of 1.483 provided a 90% confidence level for the False Discovery Rate (FDR). The TAIR 8 Microarray Elements Search website (http://www.arabidopsis.org/tools/bulk/microarray/index.jsp) was used to determine which gene(s) each Affymetrix probe was assigned to (as of May 30, 2008). The microarray data, normalized by MAS5.0 to a TGT value of 500, are available on the Bio-Array Resource website as Project 1 of the Project Browser at http://bar.utoronto.ca/affydb/cgi-bin/affy_db_proj_browser.cgi, under the identifiers bot0004, bot0006, and bot0008 for the mock inoculated samples; and bot0005, bot0007, and bot0009 for the *Pst*-inoculated samples. Affymetrix CEL files are available upon request.

2.6d Identification of T-DNA Insertion Mutants

PCR screening employed a three-primer PCR strategy that identified individuals with wild-type, heterozygous, and homozygous T-DNA insertions in a single step. Gene-specific primers in combination with the LBb1 primer (The Institute for Molecular Biology and Biotechnology – MOBIX: Table 2.3) were used as described on the T-DNA Express Web site (http://www.signal.salk.edu/tdna_FAQs.html). Plants homozygous for T-DNA insertions in *ANAC055, ANAC092, CDA1*, and *UGT85A1* were used for further analysis.

2.6e Gene Expression Using RT-PCR

Leaf samples were collected from Col-0 inoculated with *Pst* to analyze gene expression between 0 and 48 hours post inoculation (hpi) from young (3 wpg) and mature (5 or 6 wpg) plants. RNA was extracted from leaf samples using the TRIzol method

(Invitrogen), DNase treatment was performed with the DNase Free (Ambion) system, and cDNA was synthesized using the SuperScript III (Invitrogen) reverse transcriptase kit, according to the manufacturers' instructions. Tests were carried out for each set of gene-specific primers (The Institute for Molecular Biology and Biotechnology - MOBIX : Table 2.3) to determine the cycle number giving logarithmic amplification (26-28 PCR cycles in this work). Actin was used as the constitutive internal control. All gels were stained with ethidium bromide, except the gel in Fig 2.3b which was stained with SYBR Gold (Invitrogen).

2.6f Bioinformatics Analysis

Promoter analysis of *ANAC055*, *ANAC092*, *CDA1*, and *UGT85A1* was conducted with the *Arabidopsis* Cis-Regulatory Database (AtCisDB) at the Ohio State University *Arabidopsis* Gene Regulator Information Server (AGRIS: http://arabidopsis.med.ohio-state.edu/AtcisDB/) (default settings) (Davuluri et al., 2003).

A rooted dendrogram was produced by the CLUSTAL W multiple alignment analysis tool (Thompson et al., 1994) of the San Diego Super Computer (http://workbench.sdsc.edu/) (default settings) with the deduced amino acid sequences of ANAC055, ANAC092, ANAC100 (At5g61430), ANAC079 (At5g07680), ANAC074 (At4g28530), ANAC019 (At1g52890), ANAC072 (At4g27410), ANAC102 (At5g63790), ANAC025 (At1g61110), ANAC009 (At1g26870), ANAC040 (At2g27300), ANAC062 (At3g49530), ANAC064 (At3g56530), ANAC050 (At3g10480), ANAC077 (At5g04400), ANAC083 (At5g13180), ANAC037 (At2g18060), ANAC090 (At5g22380), ANAC045 (At3g03200), BnNAC1-1

(AY245879), BnNAC5-1 (AY245881), BnNAC3 (AY245880), BnNAC5-7 (AY245882), BnNAC5-8 (AY245883), BnNAC5-11 (AY245884), BnNAC18 (AY245885), BnNAC14 (AY245886), and BnNAC485 (AY245887) .

2.7 Acknowledgements

We thank ABRC for T-DNA insertion lines.

Chapter 3:

Two NAC transcription factors, ANAC055 and ANAC092, play non-redundant roles in an EIN2-dependent pathway during age-related resistance in Arabidopsis

3.1 Preface

This chapter is being prepared for publication. M. Melas performed one replicate of the ARR experiment in Fig 3.7A. All other experiments were performed by F. Al-Daoud.

3.2 Abstract

As *Arabidopsis thaliana* matures it exhibits age-related resistance (ARR) to virulent *Pseudomonas syringae*. ARR is associated with flowering, intercellular accumulation of salicylic acid (SA), and expression of some jasmonic acid/ethylene (JA/ET)-associated genes. Here, the role of two No Apical Meristem Cup-shaped Cotyledon (NAC) transcription factors, ANAC055 and ANAC092, was studied by characterizing a number of ANAC055, ANAC092, and JA/ET signaling mutants. The ARR defect of *anac092* is followed one week later by the onset of an enhanced ARR response, and this is associated with delayed flowering. *ANAC092*-overexpressing plants display reduced ARR that is associated with decreased expression of the *PDF1.2a* JA/ET defense gene. Taken together, this suggests that ANAC092 negatively regulates JA/ET signaling during ARR and promotes to the onset of ARR and flowering. Also, the flowering-delayed mutant *luminidependens1* (*ld-1*) exhibits an ARR defect, suggesting that LD contributes to ARR. ANAC055 seems to be a positive regulator of JA/ET signaling during ARR, as reduced ARR in *anac055* is associated with decreased expression of *PDF1.2a*. *anac055anac092* double mutant analysis supports the idea that ANAC055 and ANAC092 play non-redundant roles during ARR. Moreover, *ethylene*

insensitive2-1 (*ein2-1*) exhibits an ARR defect and attenuated expression of both *NAC*s, suggesting that the *NAC*s are expressed in an EIN2-dependent manner during ARR.

3.3 Introduction

Resistance to infection by pathogens varies as plants age. Resistance to some pathogens increases as some plants mature, and in other cases resistance to pathogens decreases with plant age. Development of resistance to pathogens with age is known as age-related resistance (ARR). The onset of ARR in some plants is associated with flowering, senescence, or stressful growing conditions (Panter and Jones, 2002; Whalen, 2005; Develey-Riviere and Galiana, 2007). The molecular mechanism of ARR in many plants is not known, but ARR in tobacco has been studied in some detail. As tobacco matures it becomes more resistant to *Peronospora tabacina* (Wyatt et al., 1991), *Phytophthora parasitica* (Hugot et al., 1999), and Tobacco Mosaic Virus (TMV: Fraser, 1981; Yalpani et al., 1991; Yalpani et al., 1993). ARR in tobacco is associated with flowering, accumulation of *Pathogenesis Related* (*PR*) gene transcripts, and accumulation of anti-microbial compounds in mature leaves (Fraser, 1981; Wyatt et al., 1991; Yalpani et al., 1993; Hugot et al., 1999; Hugot et al., 2004). Mature tobacco leaves inhibit initial infection by *P. parasitica* in a salicylic acid (SA)-independent manner, and they utilize SA-dependent mechanisms to repress its subsequent spread (Hugot et al., 1999). Therefore, there seems to be SA-dependent and SA-independent ARR pathways in tobacco.

Young Arabidopsis (*Arabidopsis thaliana*) plants initiate a resistance response known as basal resistance or PAMP-triggered immunity (PTI) when they are infected

with the virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 strain. PTI is one of the first lines of pathogen defense triggered by perception of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) by Arabidopsis pattern recognition receptors (PRRs: Boller and He, 2009). To counter PTI, *Pst* evolved a number of mechanisms that inhibit plant defense by utilizing phytotoxins, such as coronatine, that are secreted in the plant's intercellular space, and protein effectors that are delivered via its type III secretion system (TTSS: Boller and He, 2009). This allows *Pst* to colonize Arabidopsis more successfully (in greater numbers).

Resistance to some pathogens changes as Arabidopsis ages. Mature Arabidopsis exhibits ARR to virulent and avirulent *Pst* (Kus et al., 2002; Cameron and Zaton, 2004; Rusterucci et al., 2005; Carviel et al., 2009), *Ps* pv. *maculicola* (*Psm*; Kus et al., 2002), *P. viridiflava* (*Pv*: Goss and Bergelson, 2006), the oomycete *Hyaloperonospora arabidopsidis* (McDowell et al., 2005; Rusterucci et al., 2005), and *Cauliflower Mosaic Virus* (CaMV: Leisner et al., 1993). There is a significant 10- to 100-fold decrease in virulent *Pst* DC3000 (herein referred to as *Pst*) levels associated with ARR in mature Arabidopsis plants (~6 wpg) compared to young plants (3 to 4 wpg) grown under short days (Kus et al., 2002). Arabidopsis is thought to exhibit ARR in association with the transition to flowering in six week-old plants (Kus et al., 2002). This idea was supported by Rusterucci et al. (2005) who showed that plants grown under long day conditions that promote early flowering exhibit ARR earlier than plants grown under short day conditions that promote late flowering (Rusterucci et al., 2005). Also, Eriksson et al. (2006) showed that expression of some flowering marker genes, including *Apetala1*

(*AP1*), are up-regulated in the shoot apical meristem (SAM) of Arabidopsis at around six wpg under short days. They concluded that short day-grown Arabidopsis plants undergo the transition from vegetative to reproductive growth at six wpg (Eriksson et al., 2006). This coincides with ARR exhibited in six week-old Arabidopsis under short days (Rusterucci et al., 2005). Furthermore, ARR to CaMV is also observed earlier in some early flowering Arabidopsis plants compared to some plants that are delayed in flowering (Leisner et al., 1993; Cecchini et al., 2002). Taken together, this data suggest that the transition from vegetative to reproductive growth in Arabidopsis is important for the onset of ARR.

A number of SA accumulation-deficient plants do not exhibit ARR to *Pst*, including *NahG*, *SA induction deficient1* (*sid1*), *sid2*, *phytoalexin accumulation deficient4* (*pad4*), *enhanced disease susceptibility1* (*eds1*), and *important for ARR pathway1-1* (*iap1-1*), suggesting that SA accumulation is necessary for ARR (Kus et al., 2002; Cameron and Zaton, 2004; Carviel et al., 2009). SA usually acts as a signal during pathogen responses to up-regulate *PR* genes through activation of Non-expressor of PR genes1 (NPR1; Fobert and Despres, 2005). Expression of *PR1* is reduced in mature plants exhibiting ARR compared to young plants after inoculation with *Pst* (Kus et al., 2002; Rusterucci et al., 2005). This observation combined with the ARR-competent nature of the SA signaling-deficient *npr1* plants (Kus et al., 2002) suggests that SA does not play its usual signaling role during ARR (Kus et al., 2002; Rusterucci et al., 2005). SA levels are elevated in leaves and intercellular washing fluids (IWFs) of plants displaying ARR (Cameron and Zaton, 2004; Carviel et al., 2009). IWFs of mature plants contain more SA

than IWFs collected from young plants after inoculation with *Pst* (Cameron and Zaton, 2004). These IWFs possess anti-microbial properties (Kus et al., 2002; Cameron and Zaton, 2004), which may be the result of the anti-microbial activity of SA (Sikkema et al., 1995; Johnson et al., 2000; Choi and Gu, 2001; Cameron and Zaton, 2004; Lee et al., 2005). Infiltrating salicylate hydroxylase, an enzyme that converts SA into inert catechol, into the intercellular space of mature plants results in a reduced ARR response (Cameron and Zaton, 2004). Furthermore, infiltrating SA into the intercellular space of SA accumulation-compromised *sid2*, *pad4-1*, *eds1-1*, and *iap1-1* partially rescues their ARR defects (Cameron and Zaton, 2004; Carviel et al., 2009). Considered together, these data suggest that SA plays an anti-microbial role in the intercellular space during ARR.

Additional genes that contribute to ARR were identified in a microarray experiment in which gene expression was compared between mature, mock-inoculated Arabidopsis plants and mature plants inoculated with *Pst* (Chapter 2; Carviel et al., 2009). A number of jasmonic acid/ethylene (JA/ET)-associated genes were up-regulated during ARR, including two JA/ET-associated *No Apical Meristem Cup-shaped Cotyledon* (*NAC*) genes, *ANAC055* and *ANAC092*. T-DNA insertion mutants *anac055* and *anac092* displayed reduced ARR against *Pst* and *H. arabidopsidis*, suggesting that ANAC055 and ANAC092 play a role in ARR (Chapter 2; Carviel et al., 2009). Furthermore, *ANAC055* and *ANAC092* were differentially expressed during ARR to *Pst* in mature plants compared to young plants after inoculation with *Pst* (Chapter 2; Carviel et al., 2009). This data suggests that these JA/ET-associated NACs contribute to ARR. However, Carviel et al. (2009) also showed that the JA signaling mutants *jasmonate insensitive1-1*

(*jin1-1*) and *jasmonate resistant1-1* (*jar1-1*) were competent for ARR, suggesting that ARR does not require JIN1 or JAR1.

The Arabidopsis *NAC* gene family consists of 105 members encoding proteins with a conserved, highly charged N-terminal DNA-binding domain (NAC domain), and a variable transactivation domain at the C-terminus (Ooka et al., 2003; Olsen et al., 2005). NACs are involved in many aspects of plant development and responses to stress (Olsen et al., 2005). ANAC055 and ANAC092, for example, play roles in a number of osmotic stress responses and they are expressed in response to NaCl treatment (Tran et al., 2004; He et al., 2005). ANAC092 is also involved in leaf senescence (Kim et al., 2009). In addition, expression of these *NAC*s is up-regulated in response to a number of phytohormones: *ANAC055* is expressed in response to methyl JA application (MeJA: Tran et al., 2004; Bu et al., 2008), *ANAC092* is responsive to ET treatment (Alonso et al., 2003), and they are both expressed in response to abscisic acid (ABA) treatment (Tran et al., 2004; He et al., 2005). ANAC055 and ANAC092 have previously been implicated in defense, as their transcripts are expressed in young plants after inoculation with *Psm* (Wang et al., 2008). *Pst* type III effectors also induce expression of these *NAC*s (Truman et al., 2006; de Torres-Zabala et al., 2007). In addition, ANAC055 contributes to resistance to the necrotrophic fungus *Botrytis cinerea* and it regulates JA-induced expression of some JA-associated genes, including *Lipoxygenase2* (*LOX2*) and *Vegetative Storage Protein1* (*VSP1*: Bu et al., 2008). Expression of both *ANAC055* and *ANAC092* in young Arabidopsis plants after inoculation with *Psm* is dependent on Ethylene Insensitive2 (EIN2) (Wang et al., 2008), and *ANAC092* is expressed in an

EIN2-dependent manner in response to NaCl treatment and during leaf senescence (He et al., 2005; Kim et al., 2009). This suggests that these NACs are components of an EIN2-dependent pathway. *EIN2* encodes a member of the Nramp transmembrane protein family that is considered to be a central component of JA/ET signaling (Alonso et al., 1999). Taken together, this data suggests that ANAC055 and ANAC092 participate in JA/ET signaling.

Here, a genetics approach is utilized to explore the relationship between ANAC055 and ANAC092 and JA/ET signaling during ARR to *Pst*. The ARR phenotypes of single and double *ANAC055* and *ANAC092* T-DNA insertion knockout mutants, *ANAC092*-overexpressing plants, and *lipoxygenase2* (*lox2*: JA accumulation-deficient) and *ein2-1* (JA/ET signaling-compromised) JA/ET signaling mutants were characterized. The relationship between flowering and ARR is also explored by characterizing the ARR phenotype of the late flowering *luminidependens1* (*ld-1*) mutant.

3.4 Results

3.4a *anac055anac092* displays a similar ARR defect to *anac055* and *anac092*

Young, three week-old *anac055* and *anac092* are as susceptible to *Pst* as wild-type plants, whereas mature, six week-old *anac055* and *anac092* are partially defective in ARR to *Pst* compared to the wild-type (Chapter 2; Carviel et al., 2009). The partial ARR response displayed by *anac055* and *anac092* could be due to partial functional redundancy between ANAC055 and ANAC092. Significant functional redundancy exists between members of the NAC family that possess highly similar amino acid sequences, such that some single *NAC* gene mutants display wild-type phenotypes, while some

double and triple mutants display mutant phenotypes (Aida et al., 1997; Aida et al., 1999; Vroemen et al., 2003). To determine if ANAC055 and ANAC092 are partially functionally redundant during ARR the amino acid sequences of ANAC055 and ANAC092 were compared in a protein alignment (Clustal W), and an *anac055anac092* double mutant was created and tested for ARR (see Materials and Methods). Overall, ANAC055 and ANAC092 were 32% identical with greater identity in their putative NAC DNA-binding domains (51% identical) compared to their putative transactivation domains (10% identical; Fig 3.1A). Unlike ANAC092, a serine-rich region is present in the transactivation domain of ANAC055 (Fig 3.1A), which may be important for post-translational modification of this protein (Huber and Hardin, 2004).

If ANAC092 and ANAC055 are partially redundant during ARR, then a double mutant should exhibit a synergistic or additive phenotype compared to the single *nac* mutants. The ARR phenotype of *anac055anac092* double T-DNA insertion mutants was compared to *anac055*, *anac092*, and wild-type Columbia-0 (Col-0) plants. ARR experiments are performed on mature plants (five or six weeks old) grown under short day conditions (9 hr photoperiod) by inoculating them with *Pst* (10^6 cfu ml^{-1}). *In planta Pst* density is determined three days post inoculation (dpi). In the *anac055anac092* ARR experiment, mature Col-0 supported low levels of *Pst* (9.6×10^4 cfu ld^{-1}), typical of plants exhibiting ARR. Mature *anac055anac092* exhibited an ARR defect similar to *anac055* and *anac092*, such that *Pst* levels in *anac55anac092*, *anac055*, and *anac092* were four-fold higher than in Col-0 (ANOVA $P < 0.05$, Fig 3.1B). These data suggest that ANA055

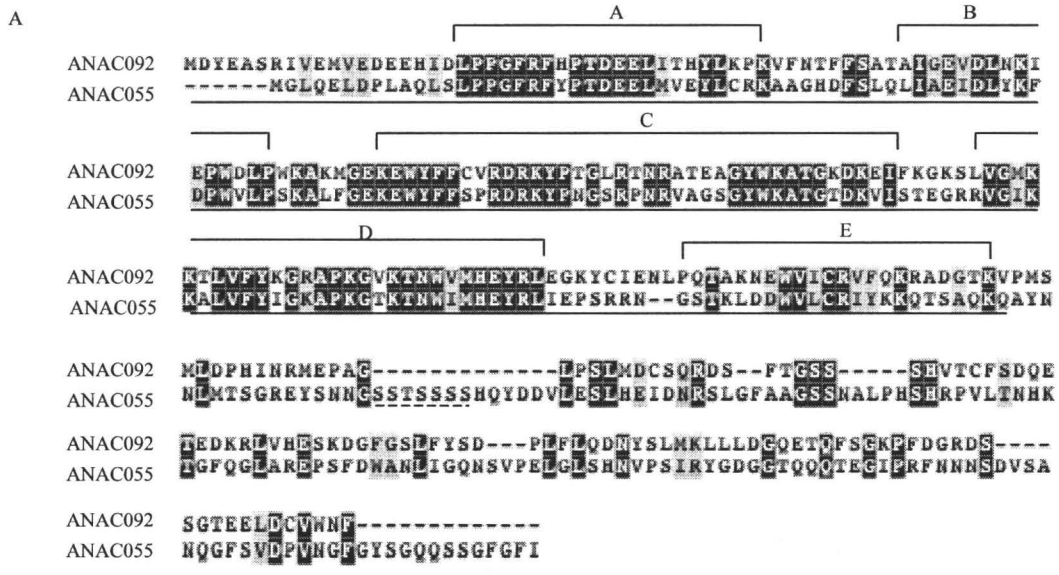
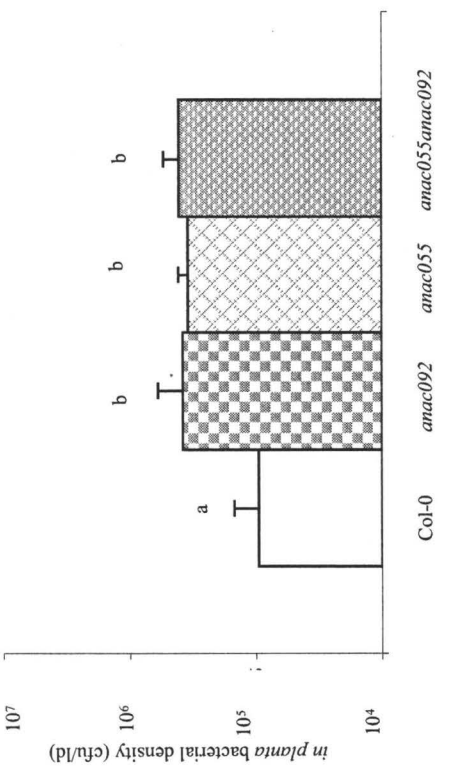


Figure 3.1. A, Multiple alignment of the deduced amino acid sequences of ANAC055 and ANAC092 with CLUSTAL W. The putative NAC DNA-binding domain is underlined, and the conserved NAC DNA-binding motifs (A-E; Ooka et al., 2003) are indicated. A serine repeat present in the transactivation domain of ANAC055 is underlined with a dashed line. Identical residues are shaded in black and similar residues are shaded in gray. B, *In planta Pst* growth in mature (5 wpg) Col-0, *anac055*, *anac092*, and *anac055anac092* (logarithmic scale). Plants were inoculated with 10^6 cfu ml^{-1} virulent *Pst DC3000,* and *Pst* growth was assayed three days post inoculation (dpi) for all experiments herein. *Pst* growth for all the experiments in this report is represented as an average of three replicates with eight leaf discs (ld) from different leaves included in each replicate. Different letters represent significant differences between mature genotypes (Tukey's Honest Significant Difference, $P < 0.05$). This experiment was performed three times with similar results (see Appendix A Fig A5 for replicates).


B
in planta bacterial density (cfu/ld)
10^7
10^6
10^5
10^4
a
b
b
b
Col-0
anac092
anac055
anac055anac092

and ANAC092 perform non-redundant functions in the same ARR pathway. Alternatively, other NACs may be playing similar roles to ANAC055 and ANAC092 during ARR, which might contribute to the lack of a synergistic phenotype in *anac055anac092*.

The microarray experiment presented in Chapter 2, where gene expression of mock-inoculated mature plants was compared to mature plants inoculated with *Pst*, identified a third *NAC* gene that was up-regulated during ARR, *ANAC029* (At1g69490: Carviel et al., 2009). ANAC029 might be functionally redundant with ANAC055 and/or ANAC092 during ARR. Previous phylogenetic analysis of NAC domains of all the known Arabidopsis NAC proteins suggests that the NAC domain of ANAC029 is more similar to ANAC055 than ANAC092 (Ooka et al., 2003). Overall, ANAC029 is 37% identical to ANAC055 and 36% identical to ANAC092 (Fig 3.2). Additionally, other members of the NAC family that are more similar to ANAC055 and ANAC092 may play a partially redundant role with them during ARR. A BLAST search with the full putative amino acid sequence of the NACs identified ANAC019 (At1g52890) as the most similar NAC protein to ANAC055 (67% identity), and ANAC059 (At3g29035) was the most similar NAC protein to ANAC092 (58% identity) (Fig 3.3). This is consistent with the previously mentioned phylogenetic analysis of all known Arabidopsis NAC domains performed by Ooka et al. (2003). This phylogenetic analysis also showed that ANAC072 (At4g27410) and ANAC047 (At3g04070) clustered with ANAC055, and ANAC079 (At5g07680) and ANAC100 (At5g61430) clustered with ANAC092 (Ooka et al., 2003). Therefore, ANAC019, ANAC072, and/or ANAC047 may be partially redundant with

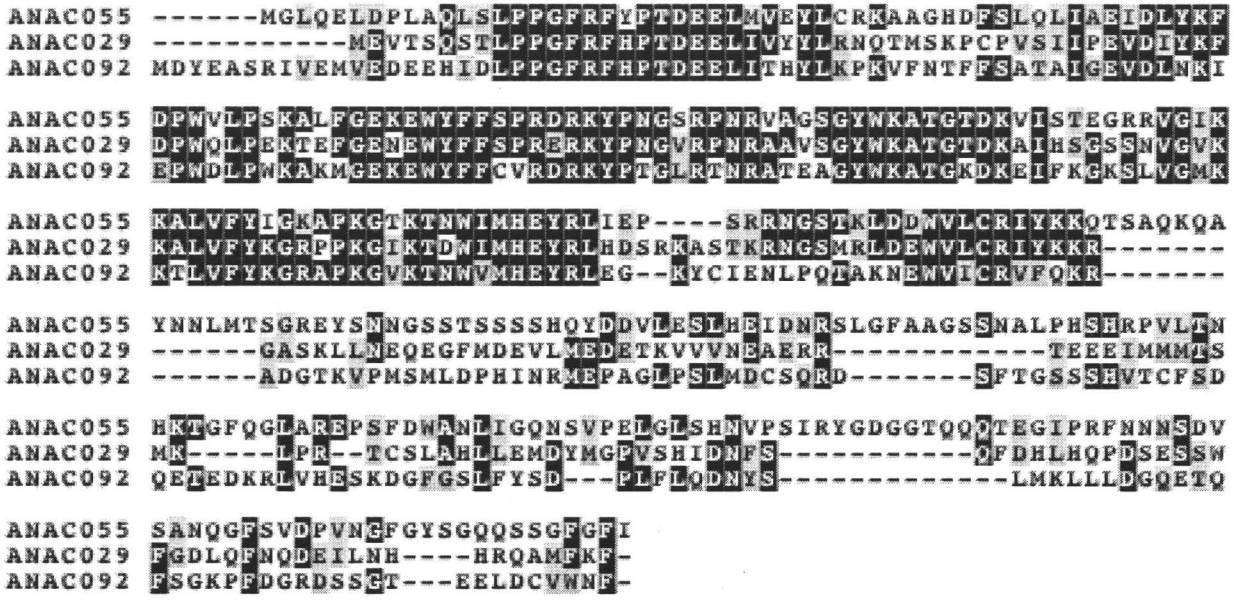


Figure 3.2. Multiple alignment of the deduced amino acid sequences of ANAC055, ANAC092, and ANAC029 with CLUSTAL W. Identical residues are shaded in black and similar residues are shaded in gray.

Figure 3.3. A, Multiple alignment of the deduced amino acid sequences of ANAC055 and ANAC019, and B, ANAC092 and ANAC059 with CLUSTAL W. Identical residues are shaded in black and similar residues are shaded in gray.

ANAC055, and ANAC059, ANAC079, and/or ANAC100 may be partially redundant with ANAC092 during ARR.

3.4b The ARR defect of *anac055* is age-specific

anac055 displays a reduced ARR response at six wpg (Chapter 2; Carviel et al., 2009). ARR also occurs at other ages of Arabidopsis, including seven wpg (Kus et al., 2002). ANAC055 may contribute to ARR not only at six wpg, but also at other ages of Arabidopsis. To test this idea, ARR assays were performed with Col-0 and *anac055* at different ages (3 to 7 wpg; Fig 3.4A). As Col-0 matured it gradually became more resistant to *Pst*. Young plants supported high levels of *Pst* ($> 1 \times 10^6$ cfu ld^{-1}) at three and four wpg. At five wpg, Col-0 supported an intermediate level of *Pst* (5×10^5 to 1×10^6 cfu ld^{-1}), whereas it supported low levels of *Pst* ($< 5 \times 10^5$ cfu ld^{-1}) at six and seven wpg. There was a 35-fold decrease in *Pst* levels in mature, six week-old Col-0 compared to young plants at three wpg (Student's t-test $P < 0.05$; Fig 3.4A). *anac055* supported similar levels of *Pst* to Col-0 at three, four, five, and seven wpg, whereas at six wpg *Pst* levels were three-fold higher in *anac055* compared to Col-0 (Student's t-test $P < 0.05$; Fig 3.4A). Thus it appears that the *anac055* ARR defect is observed only in six week-old plants.

3.4c *anac092* exhibits delayed flowering associated with delayed onset of an enhanced ARR response

While performing ARR experiments it was observed that fewer *anac092* plants had inflorescence stems than wild-type plants at six wpg, suggesting that *anac092* flowered later than Col-0. Since ARR is associated with flowering (Rusterucci et al.,

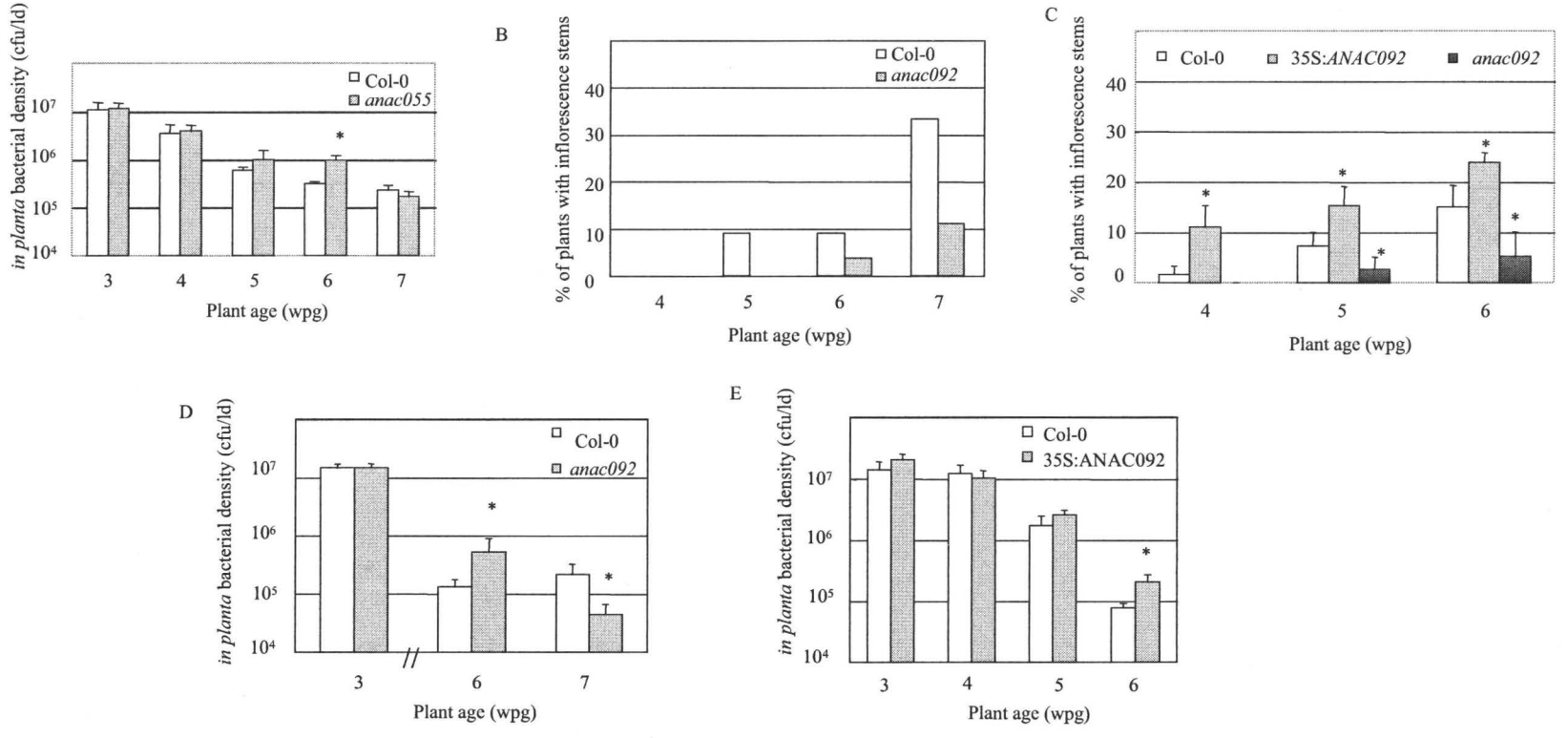


Figure 3.4. A, *In planta Pst* growth (logarithmic scale) in Col-0 and *anac055* plants at 3 to 7 wpg. B, The % of plants with macroscopically visible inflorescences was determined for Col-0 and *anac092* at 4 to 7 wpg (n ≥18 plants). C, The % of plants with macroscopically visible inflorescences was determined for Col-0, *anac092*, and 35S:*ANAC092* plants at 4 to 6 wpg. This is presented as an average of three replicates +/- SD with at least 18 plants in each replicate. D, *In planta Pst* growth in Col-0 and *anac092* (same plants in part B) at 3, 6, and 7 wpg. E, *In planta Pst* growth in Col-0 and 35S:*ANAC092* at 3 to 6 wpg. * $P < 0.05$ Student's T-Test between wild-type and each mutant plant. These experiments were performed at least three times with similar results (see Appendix A Fig A6 and Fig A8 for replicates). For details of *Pst* quantification refer to the legend of Fig 3.1.

2005), then the delayed flowering phenotype of *anac092* may delay the onset of ARR. To test this idea, the flowering time of Col-0 and *anac092* was determined and ARR experiments were performed at different ages. The flowering time was measured by calculating the percent of plants with macroscopically visible inflorescence stems at four to seven wpg (Fig 3.4B,C), as previously done by Rusterucci et al. (2005). Less than 10% of young Col-0 and *anac092* plants possessed inflorescence stems at four and five wpg. Inflorescence stems were visible for less than 20% of Col-0 at six wpg, whereas less than 10% of *anac092* had inflorescence stems at six wpg (similar to younger plants) (Student's t-test $P < 0.05$; Fig 3.4B,C). At seven wpg, 33% of wild-type plants possessed an inflorescence stem, but only 11% of *anac092* plants had inflorescence stems (similar to 6 week-old Col-0; Fig 3.4B). The data illustrates that fewer *anac092* plants have visible inflorescence stems compared to Col-0, suggesting that *anac092* flowers later than the wild-type.

If delayed flowering in *anac092* delays the onset of ARR, then the ARR defect in six week-old *anac092* might be followed by a wild-type ARR response at seven wpg. To test this prediction, ARR assays were performed on Col-0 and *anac092* at three, six, and seven wpg. During this experiment, Col-0 was 114-fold more resistant to *Pst* at six wpg compared to three wpg (Student's t-test $P < 0.05$; Fig 3.4D), and there was little difference in *Pst* levels at six and seven wpg (Fig 3.4D). Young *anac092* was as susceptible to *Pst* as wild-type plants, while six week-old *anac092* supported four-fold higher *Pst* levels than Col-0 (Student's t-test $P < 0.05$; Fig 3.4D). Interestingly, at seven wpg *Pst* levels were five–fold lower in *anac092* compared to Col-0 (Student's t-test $P <$

0.05; Fig 3.4D). Therefore, *anac092* displays reduced ARR at six wpg and an enhanced ARR response at seven wpg.

Collectively, this ARR and flowering time data suggest that the ARR defect at six wpg and enhanced ARR response at seven wpg displayed by *anac092* are associated with a delay in flowering. Since the onset of ARR is associated with flowering (Rusterucci et al., 2005), the delay in flowering of *anac092* may be delaying the onset of an enhanced ARR response such that an ARR defect is observed at six wpg and an enhanced ARR response is observed at seven wpg. This suggests that ANAC092 is a positive regulator of flowering and the onset of ARR, and a negative regulator of ARR. Unlike *anac092*, *anac055* did not display a delayed flowering phenotype associated with its ARR defect (data not shown), suggesting that the ARR defect in *anac055* is not the result of a delay in the onset of ARR.

3.4d *ANAC092*-overexpressing plants exhibit reduced ARR

If ANAC092 is a negative regulator of ARR, then *ANAC092*-overexpressing plants may exhibit a reduced ARR response. To test this prediction, *ANAC092*-overexpressing plants (35S:*ANAC092*: He et al., 2005) were tested for ARR in weekly assays (Fig 3.4E). Col-0 sometimes exhibits a gradual onset of ARR and other times it displays an abrupt onset of ARR (Kus et al., 2002; Rusterucci et al., 2005). During the course of this experiment, Col-0 displayed an abrupt onset of ARR as it matured compared to some other experiments (compare Fig 3.4A and Fig 3.4E). In Fig 3.4A Col-0 exhibited a gradual reduction in *Pst* levels with age, whereas in Fig 3.4E plants displayed an abrupt onset of ARR as young Col-0 (three to five wpg) supported high *Pst* levels (>

10^6 cfu ld^{-1}), and mature, six-week old plants supported low levels of *Pst* typical of plants exhibiting ARR (< 5 x 10^5 cfu ld^{-1}; Fig 3.4E). Similar to Col-0, 35S:*ANAC092* supported high levels of *Pst* (> 10^6 cfu ld^{-1}) at three, four, and five wpg, whereas six week-old 35S:*ANAC092* supported three–fold higher *Pst* levels than Col-0 (Student's t-test $P <$ 0.05; Fig 3.4E). This suggests that overexpression of *ANAC092* reduces the ARR response to *Pst*, thereby lending support to the idea that ANAC092 is a negative regulator of ARR. Furthermore, mature 35S:*ANAC092* plants were 100-fold more resistant to *Pst* compared to young 35S:*ANAC092* plants (Student's t-test $P < 0.05$; Fig 3.4E) (six week-old Col-0 was 177-fold more resistant to *Pst* than three-week old Col-0), suggesting that these plants possess a partial ARR response, as do *anac055* and *anac092* (Fig 3.4A,D; Chapter 2; Carviel et al., 2009).

If ANAC092 is a positive regulator of the onset of ARR and the transition to flowering, then 35S:*ANAC092* may flower earlier than wild-type plants. Therefore, the flowering time of Col-0 and 35S:*ANAC092* was determined during weekly ARR assays as previously described. While less than 10% of young wild-type plants had inflorescence stems at four and five wpg, 10 to 25% of young 35S:*ANAC092* plants had inflorescence stems at four and five wpg (Student's t-test $P < 0.05$; Fig 3.4C). At six wpg, less than 20% of wild-type plants possessed inflorescence stems, whereas 20 to 25% of 35S:*ANAC092* had inflorescence stems (Student's t-test $P < 0.05$; Fig 3C). This data suggests that 35S:*ANAC092* underwent the transition to flowering earlier than wild-type plants during these weekly experiments. Curiously, the early appearance of bolts in 35S:*ANAC092* was not associated with an altered timing of the onset of ARR, as young

plants supported similar levels of *Pst* as Col-0 (Fig 3.4E). Therefore, while the absence of *ANAC092* is associated with a delay in flowering and the onset of an enhanced ARR response, overexpression of *ANAC092* is associated with reduced ARR and accelerated flowering. This supports that idea that ANAC092 is a negative regulator of ARR, and a positive regulator of flowering and the onset of ARR. However, overexpressing *ANAC092* does not appear to be sufficient to accelerate the onset of ARR. Other factors may be required, possibly in combination with ANAC092, to initiate the onset of ARR in Arabidopsis.

3.4e Expression of some defense genes is reduced in *anac055* and 35S:*ANAC092*

ANAC055 and ANAC092 have been shown to be functional transcription factors involved in JA/ET signaling (Tran et al., 2004; He et al., 2005; Bu et al., 2008; Wang et al., 2008). Additionally, a number of JA/ET-associated genes were up-regulated in the ARR microarray suggesting that JA/ET signaling occurs during ARR (Chapter 2; Carviel et al., 2009). ANAC055 and ANAC092 may regulate JA/ET gene expression during ARR. To test this hypothesis, expression of the JA/ET-associated genes *Lipoxygnease2* (*LOX2*: JA biosynthesis gene) and *Plant Defensin1.2a* (*PDF1.2a*: JA/ET signaling marker gene) was determined in mature *anac055*, 35S:*ANAC092*, and Col-0 during ARR (gene primers listed in Table 3.1). Since SA is important for ARR (Kus et al., 2002; Cameron and Zaton, 2004, Carviel et al., 2009) expression of the SA biosynthesis gene *Isochorismate Synthase1* (*ICS1*) was also monitored in these plants. In addition, expression of *ANAC092* was monitored in 35S:*ANAC092* to confirm overexpression of *ANAC092* in these plants, and expression of *ANAC055* was monitored in *anac055* to

confirm that these plants had reduced *ANAC055* expression. Leaf samples were collected from mature Col-0 plants (6 wpg) that supported low levels of *Pst* typical of plants exhibiting ARR (< 5 x 10^5 cfu ld^{-1}), and from *anac055* and 35S:*ANAC092* plants that supported at least a two-fold higher *Pst* levels compared to the wild-type.

Mature 35S:*ANAC092* expressed higher levels of *ANAC092* in untreated leaves compared to wild-type plants, confirming what was observed previously (He et al., 2005). However, 35S:*ANAC092* expressed similar levels of *ANAC092* as the wild-type at 24 hpi with *Pst* (Fig 3.5A). As expected, mature *anac055* plants expressed little *ANAC055* compared to wild-type plants at 24 hpi (Fig 3.5B), confirming that *anac055* expressed *ANAC055* to a lower level than Col-0 (Chapter 2; Carviel et al., 2009). *ICS1*, *LOX2*, and *PDF1.2a* were expressed to higher levels at 24 hpi compared to untreated leaves of ARR-competent Col-0 (Fig 3.5A), indicating that these genes are up-regulated during ARR. This was consistent with a previous study that reported up-regulation of *ICS1* during ARR (Rusterucci et al., 2005). In 35S:*ANAC092*, expression of *LOX2* and *PDF1.2a* was reduced, while *ICS1* was expressed to similar levels compared to Col-0 (Fig 3.5A). In

Table 3.1. Forward and reverse primer sequences used in RT-PCR

Gene name and AGI number	Primer sequences 5' → 3'
ANAC055 At3g15500	F[a] ATGGGTCTCCAAGAGCTTGA
	R[b] TCAAATAAACCCGAACCCAC
ANAC092 At5g39610	F ATGGATTACGAGGCATCAAG
	R TCAGAAATTCCAAACGCAAT
ICS1 At1g74710	F AACCAGTCCGAAAGACGACCTC
	R CAAATTCACTCTCCTCGCCACC
PR1 At2g14610	F AGACGCCAGACAAGTCACCGCTAC
	R TCCCTCGAAAGCTCAAGATAGCCC
LOX2 At3g45140	F ATGTATTGTAGAGAGAGTCCT
	R GGAGAAGAATATCCGCTTGGTT
PDF1.2a At5g44420	F GTTCTCTTTGCTGCTTTCGAC
	R TTATTGTAACAACAACGGGAAAAT
ACTIN1 At2g37620	F GGCGATGAAGCTCAATCCAAACG
	R GGTCACGACCAGCAAGATCAAGACG
LBb1[c]	F GCGTGGACCGCTTGCTGCAACT

[a] forward primer sequence
[b] reverse primer sequence
[c] T-DNA left border primer sequence

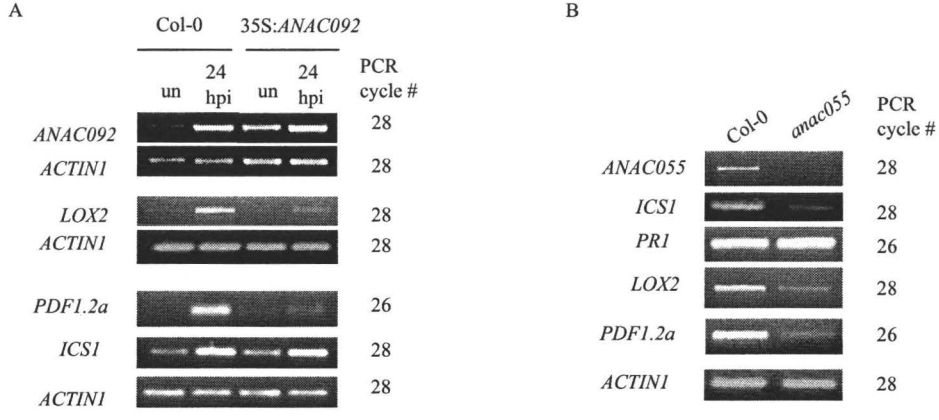


Figure 3.5. A, *ANAC092*, *LOX2*, *PDF1.2a*, and *ICS1* expression in leaves collected from untreated (un) mature (6 wpg) Col-0 and 35S:*ANAC092* plants and at 24 hpi with *Pst* (10^6 cfu ml^{-1}). B, *ANAC055*, *ICS1*, *PR1*, *LOX2*, and *PDF1.2a* expression in leaves collected from mature (6 wpg) Col-0 and *anac055* plants at 24 hpi with *Pst* (10^6 cfu ml^{-1}). *ACTIN1* is included as a loading control. The number of PCR cycles used is shown. These experiments were performed at least three times with similar results (see Appendix A Fig A27, Fig A28, and Fig A29 for replicates).

anac055, on the other hand, *ICS1*, *LOX2*, and *PDF1.2a* were all expressed to lower levels compared to Col-0 during ARR (Fig 3.5B). The attenuated expression of *ICS1* in *anac055* may be associated with decreased expression of the SA signaling marker gene *PR1*. Therefore, expression of *PR1* was tested in *anac055* and Col-0. *PR1* was found to be expressed to similar levels in *anac055* and Col-0 at 24 hpi (Fig 3.5B), suggesting that ANAC055 does not regulate this SA signaling marker gene during ARR. Taken together, this data suggests that the ARR defect observed in 35S:*ANAC092* is associated with reduced expression of *LOX2* and *PDF1.2a*, while the ARR defect of *anac055* is associated with reduced expression of *LOX2*, *PDF1.2a*, and *ICS1*. Therefore, ANAC055 may be a positive regulator of expression of *LOX2*, *PDF1.2a*, and *ICS1*, whereas ANAC092 may be a negative regulator of expression of *LOX2* and *PDF1.2a*.

3.4f *PDF1.2a* is expressed earlier and to elevated levels in mature compared to young plants after inoculation with *Pst*

ANAC092 is involved in JA/ET signaling (He et al., 2005; Wang et al., 2008), and it is expressed earlier in mature compared to young plants after inoculation with *Pst* (Chapter 2; Carviel et al., 2009). This suggests that some JA/ET-associated gene expression is up-regulated in mature plants compared to young plants after inoculation with *Pst*. To investigate this possibility, expression of the JA/ET signaling marker gene *PDF1.2a* was monitored in leaves collected from young (3 or 4 wpg) and mature (6 wpg) plants at less than 5 minutes post inoculation, 12, 24, and 48 hpi with *Pst*. Young plants supported high levels of *Pst* ($> 10^6$ cfu ld^{-1}), and mature plants supported low levels of *Pst* typical of plants exhibiting ARR ($< 5 \times 10^5$ cfu ld^{-1}). *PDF1.2a* was

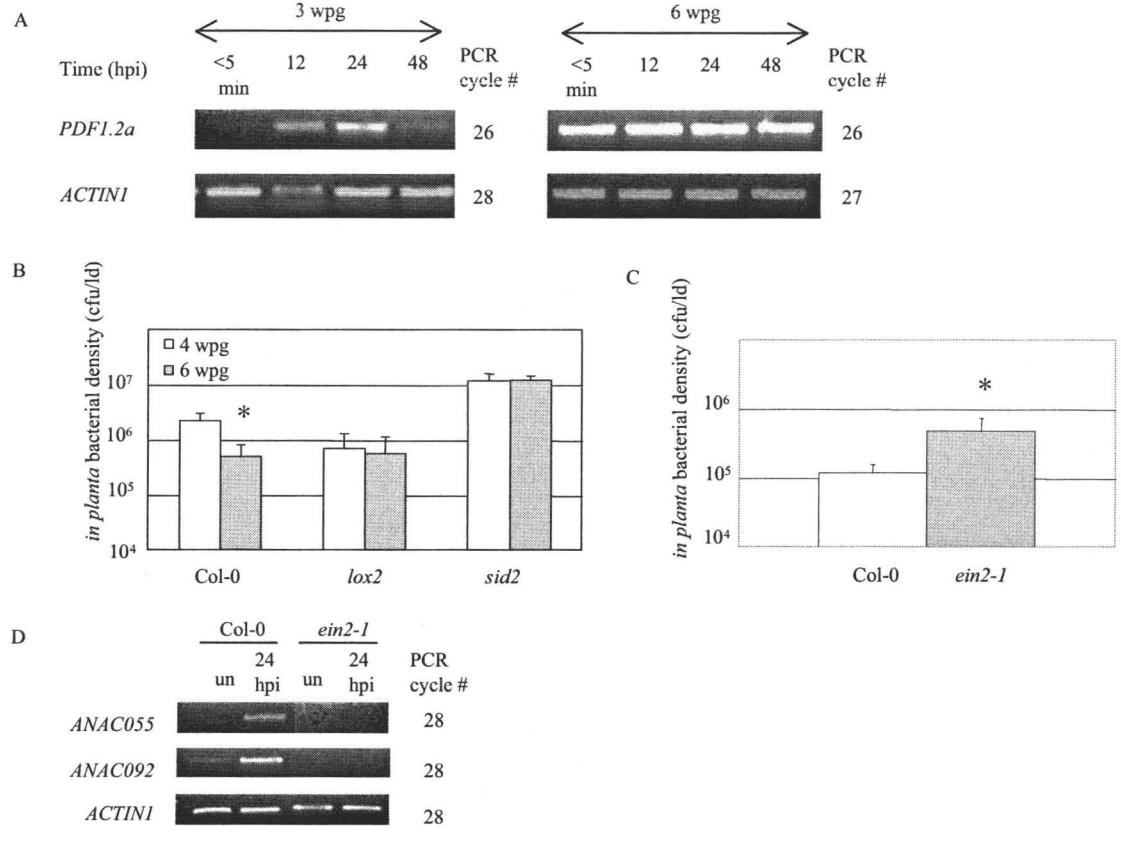


Figure 3.6. A, *PDF1.2a* expression in *Pst*-inoculated (10^6 cfu ml^{-1}) Col-0 leaves collected from young (3 wpg) and mature plants (6 wpg) at <5 min, 12, 24, and 48 hpi. *ACTIN1* is included as a loading control. The number of PCR cycles is shown. B, *In planta Pst* growth in young (4 wpg) and mature (6 wpg) Col-0, *lox2*, and *sid2* (logarithmic scale). * $P < 0.05$ Student's T-Test. C, *In planta Pst* growth in mature (6 wpg) Col-0 and *ein2-1* (logarithmic scale). * $P < 0.05$ Student's T-Test. D, *ANAC055* and *ANAC092* expression in untreated leaves (un) and at 24 hpi with *Pst* (10^6 cfu ml^{-1}) in mature (6 wpg) Col-0 and *ein2-1*. *ACTIN1* is included as a loading control. The number of PCR cycles is shown. These experiments were performed at least three times with similar results (see Appendix A Fig A3, Fig A9, and Fig A31 for replicates).

expressed to low levels in young plants at 12 and 24 hpi, but it was expressed to high levels at less than 5 minutes, 12, 24, and 48 hpi during ARR (Fig 3.6A), suggesting that *PDF1.2a* is expressed very early after inoculation with *Pst* and throughout ARR in mature plants. In addition, *PDF1.2a* appears to be up-regulated during ARR in mature compared to young plants after inoculation with *Pst*.

3.4g LOX2 is not required for ARR

Early and elevated expression of *PDF1.2a* during ARR may be a result of elevated JA accumulation and JA signaling. An effective way to diminish most JA signaling is by abolishing JA accumulation. For example, *lox2* accumulates little JA (levels similar to those observed in untreated plants) in response to inoculation with *Pst* resulting in less JA signaling than the wild-type (Bell et al., 1995; Spoel et al., 2003). To determine if reduced JA accumulation and signaling would affect the ARR response, *lox2* was tested for ARR along with Col-0 and the ARR-defective *sid2*, which contains a non-functional *ICS1* and therefore accumulates little SA in response to *Pseudomonas* (Wildermuth et al., 2001). Young, four week-old *lox2* exhibited a three-fold reduction in *Pst* levels (Student's t-test $P < 0.05$), whereas *sid2* supported five-fold greater *Pst* levels compared to wild-type plants (Student's t-test $P < 0.05$; Fig 3.6B). This demonstrated that while young *sid2* is more susceptible to *Pst* than Col-0, as previously reported (Nawrath and Metraux, 1999; Wildermuth et al. 2001), young *lox2* exhibits enhanced resistance to *Pst* compared to the wild-type. This is similar to the enhanced resistance to *P. aeruginosa* displayed by *lox2* (Prithiviraj et al., 2005), and enhanced resistance to *Pst* displayed by other JA accumulation-deficient plants (Raacke et al., 2006). As expected,

mature Col-0 (6 wpg) displayed an ARR response, whereas *sid2* did not as demonstrated by high *Pst* levels in young and mature plants. Young and mature *lox2* supported similar levels of *Pst*, and mature *lox2* exhibited similar levels of resistance as mature Col-0 (Fig 3.6B). This data suggests that LOX2-dependent JA accumulation and signaling is not required for ARR.

3.4h EIN2 contributes to ARR and regulates expression of *ANAC055* and *ANAC092*

ANAC055 and *ANAC092* are expressed in an EIN2-dependent manner in young plants after inoculation with *Psm* (Wang et al., 2008), suggesting that EIN2 might regulate expression of *ANAC055* and *ANAC092* during ARR in mature plants. To determine if EIN2 is required for ARR, *ein2-1* was included in ARR assays with Col-0. Mature, six week-old Col-0 supported low levels of *Pst* (1.2×10^5 cfu ld^{-1}) typical of plants exhibiting ARR, whereas mature *ein2-1* supported four-fold higher *Pst* levels than Col-0 (Student's t-test $P < 0.05$; Fig 3.6C). This suggests that EIN2-dependent JA/ET signaling contributes to ARR. The modest ARR defect in *ein2-1* indicates that EIN2 regulates some, but not all components of the ARR pathway. As EIN2 might regulate expression of the *NAC*s during ARR, *ANAC055* and *ANAC092* gene expression was determined in leaves of mature (6 wpg) Col-0 and *ein2-1* (untreated and at 24 hpi with *Pst*). *ANAC055* and *ANAC092* were expressed at 24 hpi during ARR in Col-0, as previously reported (Chapter 2; Carviel et al., 2009), but they were expressed to lower levels in *ein2-1* (Fig 3.6D). This suggests that expression of *ANAC055* and *ANAC092* is regulated by EIN2 during ARR.

3.4i The *luminidependens-1* late flowering mutant exhibits reduced ARR

ARR in Arabidopsis is observed earlier in some plants that flower early and later when flowering is delayed, illustrating a relationship between ARR and the transition from vegetative to reproductive growth (Leisner et al., 1993; Cecchini et al., 2002; Rusterucci et al., 2005; Goss and Bergelson, 2006). There are four main pathways that initiate flowering in Arabidopsis. The photoperiod pathway initiates flowering when plants are grown in long day conditions or are exposed to a short period of long days. The vernalization pathway initiates flowering in response to low temperatures. The gibberellin (GA) pathway initiates flowering under short day conditions, and the autonomous pathway initiates flowering under short and long days (Koorneef et al., 1998; Lee et al., 2006; Levy and Dean, 1998; Simpson and Dean, 2002). Since the onset of ARR is associated with flowering (Rusterucci et al., 2005), genes that regulate the transition to flowering might also regulate the onset of ARR. For example, components of the GA and autonomous flowering pathways might contribute to the onset of ARR under short days.

Luminidependens (*LD*) encodes for a putative transcription factor that is a regulator of expression of a number of flowering time genes, including the repressor *Flowering Locus C*, in the autonomous pathway (Lee et al., 1994; Aukerman et al., 1999; Domagalska et al., 2007). The compromised autonomous flowering pathway in *ld* plants results in delayed flowering under short and long day conditions compared to wild-type plants (Redei, 1962; Lee et al., 1994; Aukerman et al., 1999; Domagalska et al., 2007). The delayed flowering phenotype of *ld-1* might be associated with a delayed ARR

response, and this might result in an ARR defect at six wpg in *ld-1* compared to wild-type plants similar to what was observed in *anac092* (Fig 3.4D). To determine if *ld-1* exhibits an ARR defect, it was included in ARR assays with Col-0 and the ARR-defective *sid2* at six wpg (Fig 3.7A). Col-0 supported low levels of *Pst* (2.1×10^5 cfu ld^{-1}) typical of plants exhibiting ARR, while *sid2* supported forty-fold greater *Pst* levels than Col-0. *Pst* levels were elevated four-fold in *ld-1* compared to the wild-type ($P < 0.05$ Student's T-test: Fig 3.7A). During this experiment 24 +/- 7.4 % of Col-0 had inflorescence stems at six wpg, whereas none of the *ld-1* plants had inflorescence stems at that age, confirming that these plants undergo the transition to flowering later than the wild-type when grown under short day conditions (Lee et al., 1994). Taken together, this data suggests that *ld-1* exhibits a partial ARR defect associated with delayed flowering.

3.4j Long day-induced flowering in young Arabidopsis is associated with an ARR-like response

Rusterucci et al. (2005) demonstrated that plants grown under conditions that induce early flowering (long days) exhibit ARR at four wpg, while plants grown under conditions that delay flowering (short days) exhibit ARR later at six wpg. Plants grown under long days have an accelerated rate of development compared to plants grown under short days, therefore the early onset of ARR and flowering under long days may be a result of accelerated development. Since Arabidopsis is a facultative long day plant, early flowering can be induced in young plants grown under short days via the photoperiod pathway by exposing them to at least three long days (Koorneef et al., 1998; Levy and

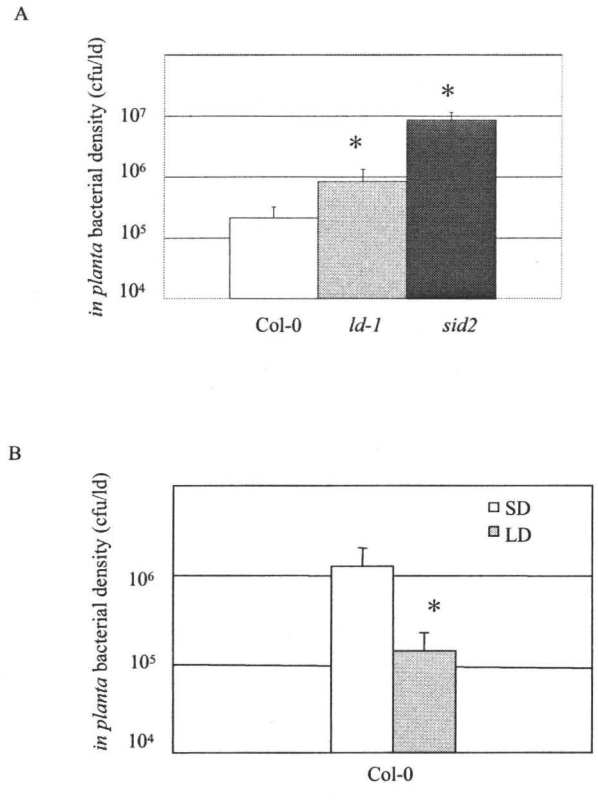


Figure 3.7. A, *In planta Pst* growth in Col-0, *ld-1*, and *sid2* at 6 wpg (logarithmic scale). This experiment was performed two times with similar results. B, *In planta Pst* growth (logarithmic scale) in 5 week-old Col-0 grown under short days (SD, 9 hr photoperiod) or exposed to 6 long days (LD, 16 hr photoperiod) at 3 wpg to initiate flowering. * $P < 0.05$ Student's T-Test. This experiment was performed two times with similar results (see Appendix A Fig A7 for replicates, M. Melas performed one replicate of the experiment in part A).

Dean, 1998; Simpson and Dean, 2002; Boss et al., 2004). This system can be used to determine if inducing flowering in young, ARR-incompetent plants is sufficient to induce ARR. This was accomplished by inducing flowering in young, three week-old plants grown under short days (9 hr. photoperiod) by exposing them to six long days (16 hr. photoperiod), and thereafter they were grown under short days. *In planta Pst* levels were determined in five week-old plants that were exposed to long days and compared to control plants (five week-old plants grown under continuous short days). All long day-exposed plants had visible inflorescence stems, confirming that this treatment induced the transition to flowering in these plants, whereas all the control plants used had no visible inflorescence stems. Control plants grown under continuous short day conditions supported high *Pst* levels ($> 10^6$ cfu ld^{-1}) typical of ARR-incompetent plants, whereas long day-exposed plants supported nine-fold lower *Pst* levels than the control plants (Student's t-test $P < 0.05$; Fig 3.7B). This data shows that inducing flowering in young Arabidopsis plants is associated with an increase in resistance to *Pst*. However, the nine-fold decrease in *Pst* levels observed in long day-exposed young plants compared to short day-grown young plants was less robust than the 19-fold decrease in *Pst* levels observed in six week-old mature plants grown under short days compared to the young plants grown under short days in this experiment. Therefore, the *Pst* resistance response exhibited by young plants after exposing them to long day conditions may not be ARR and will be referred to as an ARR-like response.

3.5 Discussion

3.5a The role of ANAC055 and ANAC092 in ARR

In this report we investigated the role of ANAC055 and ANAC092 in ARR by examining *ANAC092* and *ANAC055* single and double mutants, *ANAC092*-overexpressing plants, and the *ein2-1* JA/ET signaling mutant. Young *anac092* displays wild-type levels of susceptibility to *Pst* (Chapter 2; this chapter; Carviel et al., 2009), whereas mature, six week-old *anac092*, exhibits increased susceptibility to *Pst* (Chapter 2; this chapter; Carviel et al., 2009). However, at seven wpg, *anac092* displays increased resistance to *Pst* compared to wild-type plants. Furthermore, fewer *anac092* plants have visible inflorescence stems than Col-0, suggesting that *anac092* is delayed in flowering. As a whole, this data illustrates that *anac092* exhibits an ARR defect at six wpg and an enhanced ARR response at seven wpg, and this is associated with a delay in flowering. Since flowering is associated with the onset of ARR (Rusterucci et al., 2005), the delayed flowering of *anac092* may be delaying the onset of ARR, which may be resulting in the ARR defect observed at six wpg and the enhanced ARR response observed at seven wpg. Furthermore, *ANAC092*-overexpressing plants are compromised in ARR, such that mature, six week-old 35S:*ANAC092* plants are more susceptible to *Pst* than the wild-type. Collectively, these data suggest that ANAC092 is a negative regulator of ARR, and a positive regulator of the onset of flowering and ARR.

In contrast to ANAC092, ANAC055 appears to be a positive regulator of ARR, as mature, six week-old *anac055* exhibits increased susceptibility to *Pst* compared to wild-type plants. Therefore, ANAC055 and ANAC092 play different roles during ARR (Fig

3.8). Analysis of *anac055anac092* double mutants supports this idea since mature *anac055anac092* is as susceptible to *Pst* as *anac055* and *anac092*, illustrating that the NACs play non-redundant roles during ARR. Functional redundancy within gene families is well documented in the literature, and there is growing evidence for gene family members that function in an opposite manner. The NPR family, for example, contains positive regulators of resistance to *Pseudomonas*, such as NPR1, and negative regulators of resistance to *Pseudomonas*, such as NPR3 and NPR4 (Dong, 2004; Zhang et al., 2006). Similarly, the WRKY family in rice also contains a negative regulator of some defense responses to bacteria, WRKY45-1, and a positive regulator of some defense responses to bacteria, WRKY 45-2 (Tao et al., 2009).

Gene expression data also strongly suggest that ANAC055 is a positive regulator of ARR and ANAC092 is a negative regulator of ARR, as the ARR defects in 35S:*ANAC092* and *anac055* are associated with misexpression of some defense genes. Reduced expression of the JA/ET-associated genes *LOX2* and *PDF1.2a* is observed in mature *anac055* and 35S:*ANAC092*, and reduced expression of the SA biosynthesis gene *ICS1* is observed in mature *anac055* compared to wild-type plants displaying ARR. This suggests that ANAC055 enhances expression of *LOX2*, *PDF1.2a*, and *ICS1* during ARR, whereas ANAC092 inhibits expression of *LOX2* and *PDF1.2a* during ARR (Fig 3.8). Since ANAC055 and ANAC092 are functional transcription factors (Tran et al., 2004; He et al., 2005), ANAC092 may be a direct negative regulator of *LOX2* and *PDF1.2a*, and ANAC055 may be a direct positive regulator of *LOX2*, *PDF.12a*, and *ICS1* during ARR. Alternatively, ANAC092 and ANAC055 may indirectly regulate these genes. Other

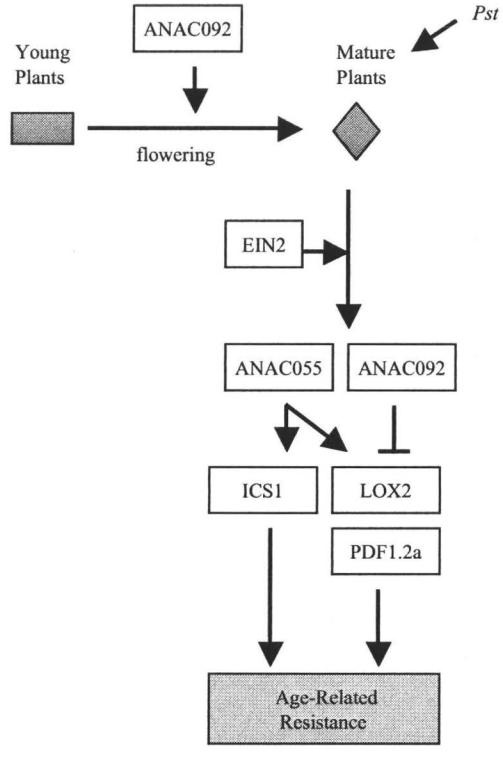


Figure 3.8. Proposed model for the role of ANAC055 and ANAC092 in the ARR pathway. As Arabidopsis ages, ANAC092 positively regulates the transition to flowering and the onset of ARR. After *Pst* inoculation, ARR-competent Arabidopsis up-regulates *ANAC055* and *ANAC092* expression in an EIN2-dependent manner. ANAC055 positively regulates expression of *ICS1, LOX2*, and *PDF1.2a*, while ANAC092 negatively regulates *LOX2* and *PDF1.2a* expression. This contributes to ARR.

NACs have also been shown to regulate defense gene expression. *ATAF1*- and *ATAF2*-overexpressing plants, for example, have reduced expression of *PR* genes and *PDF1.2a*. This is associated with increased susceptibility to the fungal pathogen *Botrytis cinerea* and *Pst* in *ATAF1*-overexpressing plants and increased susceptibility to the *Fusarium oxysporum* fungus in *ATAF2*-overexpressing plants (Delessert et al., 2005; Wang et al., 2009).

Although ANAC055 and ANAC092 seem to regulate some defense gene expression during ARR, the data suggests that they do not regulate expression of all the defense genes analyzed in this report. We show that there is wild-type expression of *ICS1* in 35S:*ANAC092* and wild-type *PR1* expression in *anac055*. This is associated with a partial ARR response in these plants, such that mature *anac055* and 35S:*ANAC092* support lower *Pst* levels compared to young plants. This suggests that other components of the ARR pathway are active in *anac055* and 35S:*ANAC092*. For example, other NACs could be partially redundant with ANAC055 and ANAC092 during ARR. If so, then these NACs may contribute to the partial ARR response in *anac055*, and overexpression of the NACs that are partially redundant with ANAC092 may be required to inhibit other ARR pathways that may be active in 35S:*ANAC092*.

The gene product of the third *NAC* gene up-regulated in the ARR microarray, *ANAC029* (Chapter 2; Carviel et al., 2009), could be partially functionally redundant with ANAC055 or ANAC092. *ANAC029* is expressed in an EIN2-dependent manner in young plants, four to five week-old Arabidopsis, after inoculation with *Psm* inoculation, similar to *ANAC055* and *ANAC092* (Wang et al., 2008). Like *ANAC055, ANAC029* is expressed

in response to MeJA treatment (McGrath et al., 2005), and ANAC029 regulates leaf senescence (Guo and Gan, 2006), as does ANAC092 (Kim et al., 2009). However, the similarity between ANAC029 and ANAC092 and ANAC055 at the amino acid level is low (less than 40% overall). Other NACs that are more similar to ANAC055 and ANAC092 could also play a partially redundant role during ARR. For example, ANAC019 (67% amino acid identity to ANAC055) is redundant with ANAC055 during defense responses to the necrotrophic fungus *Botrytis cinerea* (Bu et al., 2008). *anac055anac029*, *anac092anac029*, and *anac055anac019* double mutant analyses would further our understanding of the relationship between these NACs during ARR.

The data suggests that ANAC055 is a positive regulator of ARR (this chapter), but it is expressed to lower levels in mature plants compared to young plants after inoculation with *Pst* (Chapter 2; Carviel et al., 2009). Therefore, post-transcriptional and/or post-translational regulation may be important for its function during ARR. For example, the serine-rich region present in the transactivation domain of ANAC055 may be important for regulation of this protein. A similar mode of regulation may also be important for the *HM2* maize gene that is required for ARR against the mold pathogen *Cochliobolus carbonum* in maize plants, as its expression does not increase in mature maize compared to younger plants (Chintamanani et al., 2008).

In contrast to ANAC055, ANAC092 appears to be a negative regulator of ARR (this chapter), but it is expressed earlier in mature plants compared to young plants after inoculation with *Pst* (Chapter 2; Carviel et al., 2009). Early expression of repressors of plant defense may be a balancing mechanism that plants employ to avoid exhausting their

resources while launching an effective defense response. WRKY 11 and WRKY 17, for example, are negative regulators of basal resistance to *Pst* and their transcripts are highly expressed within 4 hpi (Journot-Catalino et al., 2006). Furthermore, early expression of other *NAC* genes in response to infection by pathogens has also been reported, including a chili pepper *NAC* that is expressed within 30 minutes of *Pseudomonas syringae* infection (Oh et al., 2005). These genes may encode important regulators of early signaling during defense responses to *Pst.*

3.5b The relationship between ARR and flowering

The ARR defects exhibited by *anac055* and 35S:*ANAC092* occur at six wpg, which coincides with the time when Arabidopsis is thought to undergo the transition from vegetative to reproductive growth under short day conditions (Eriksson et al., 2006). Therefore, ANAC055 and ANAC092 contribute to ARR when Arabidopsis is initiating flowering. To further understand the relationship between the onset of ARR and flowering time we studied the ARR response in two mutants, *ld-1* and *anac092*, that have fewer inflorescence stems at six wpg than wild-type plants and therefore are delayed in flowering. *ld-1* is an autonomous flowering pathway mutant (Lee et al., 1994; Aukerman et al., 1999), and the delayed flowering phenotype of *anac092* has not yet been characterized. Mature *anac092* and *ld-1* are more susceptible to *Pst* than wild-type plants, suggesting that they exhibit an ARR defect. Collectively, these observations suggest that the ARR defects of *anac092* and *ld-1* at six wpg are associated with delayed flowering. Therefore, the decrease in ARR in *anac092* and *ld-1* may be a consequence of delayed onset of ARR that is caused by delayed flowering, rather than a defect in ARR *per se.*

Alternatively, ANAC092 and LD might regulate both flowering and ARR independently. The enhanced ARR exhibited by *anac092* one week after it displays a reduced ARR, and the ARR defect of 35S:*ANAC092* suggest that ANAC092 contributes to negative regulation of ARR. This data strongly suggests that ANAC092 is a positive regulator of flowering time and the onset of ARR, and a negative regulator of ARR (Fig 3.8). Our data supports previous research by Rusterucci et al. (2005) that demonstrated that the onset of ARR is observed later when Arabidopsis is grown under conditions that delay flowering compared to plants that are grown under conditions that accelerate flowering. ARR to CaMV in Arabidopsis is also associated with flowering (Cecchini et al., 2002). Mature *fca-1* (autonomous pathway mutant) and *gi-2* (GA pathway mutant) flowering-delayed mutants, for example, are more symptomatic when infected with CaMV compared to wild-type plants (Cecchini et al., 2002). Similar to *anac092* and *ld-1*, the decrease in ARR in these flowering-delayed mutants is also thought to be a consequence of delayed onset of ARR (Cecchini et al., 2002).

The reduction in ARR in *anac092* and *ld-1* is modest, suggesting that they exhibit partial ARR. Other flowering pathways may be contributing to the partial ARR response observed in these mutants. LD is a component of the autonomous pathway that induces flowering under short and long days (Lee et al., 1994; Aukerman et al., 1999), and the ET-responsive nature of *ANAC092* in young plants (Alonso et al., 2003) suggests that ANAC092 may contribute to ET signaling during flowering. How ET signaling contributes to flowering is not well understood (Lin et al., 2009). A GA-dependent pathway also contributes to flowering under short days (Blazquez et al., 1997; Eriksson et

al., 2006). It may also regulate the onset of ARR, and may be responsible for the modest ARR defect observed in *anac092* and *ld-1*.

To determine if ANAC092 is sufficient to accelerate flowering and the onset of ARR we characterized the ARR response in 35S:*ANAC092*. These plants have more visible inflorescence stems than Col-0, suggesting that 35S:*ANAC092* flowers earlier than Col-0. This supports the idea that ANAC092 is a positive regulator of flowering time. However, young 35S:*ANAC092* (three to five wpg) display wild-type susceptibility to *Pst*, suggesting that the timing of the onset of ARR is not altered in these plants. Therefore, overexpression of ANAC092 appears to accelerate the transition to flowering, but it does not seem to be sufficient to initiate an early ARR response.

Onset of ARR may require activation of multiple flowering pathways. This idea is supported by the ARR-like response exhibited by short day-grown Arabidopsis after exposure to long days. Exposing short day-grown Arabidopsis to at least three long days activates a number of flowering pathways, including the photoperiod and autonomous pathways (Simpson and Dean, 2002). Plants exposed to long day conditions at three wpg display inflorescence stems earlier than plants grown under continuous short days, suggesting that they flower earlier. This is accompanied by an increase in resistance to *Pst* in plants exposed to long days compared to the control plants at five wpg. This is similar to the accelerated onset of ARR observed in early flowering Arabidopsis grown under continuous long days compared to late flowering plants grown under continuous short days (Rusterucci et al., 2005). Collectively, the 35S:*ANAC092* and long day experiments suggest that activating the ANAC092 flowering pathway accelerates

flowering time, but it may not be sufficient to induce early onset of ARR, whereas initiating a number of flowering pathways is sufficient to induce early flowering and early onset of ARR.

The data presented here suggests that *anac092* flowers later than the wild-type and this is associated with a delayed ARR response. Other studies on plants that have a mutated *ANAC092* (ethyl methanesulphonate (EMS)-mutagenized *oresara 1-1* (*ore1-1*)) have shown that these plants are also delayed in leaf senescence (under long days; Kim et al., 2009). Collectively, this data suggests that ANAC092 is a regulator of a number of plant development processes.

3.5c EIN2 contributes to ARR

One objective of this study was to identify regulators of expression of *ANAC055* and *ANAC092* during ARR. *ANAC055* and *ANAC092* are expressed in an EIN2-dependent manner in young Arabidopsis after inoculation with *Psm* (Wang et al., 2008). Here we show that mature *ein2-1* supports modestly higher *Pst* levels and reduced expression of *ANAC055* and *ANAC092* compared to wild-type plants exhibiting ARR. Therefore, EIN2 appears to be a positive regulator of expression of *ANAC055* and *ANAC092* during ARR (Fig 3.8). Previously, Kus et al. (2002) observed that the ET signaling mutant *ethylene receptor1-4* (*etr1-4*) was competent for ARR, suggesting that ETR1 is not required for ARR. *ETR1* encodes one of a number of ET receptors (Bleeker et al., 1988; Chang et al., 1993; Hua and Meyerowitz, 1998). ET is thought to be perceived by a family of receptors, including ETR1, ERS, ETR2, EIN4, and ERS2 (Bleeker et al., 1988; Chang et al., 1993; Hua and Meyerowitz, 1998). Data suggests that

in the absence of ET these receptors negatively regulate ET signaling (Hua and Meryerowitz, 1998) by activating downstream components that repress ET signaling, including the protein kinase Constitutive Triple Response1 (CTR1) (Kieber et al., 1993). ET is thought to deactivate the receptors leading to alleviation of the negative regulation, which in turn activates ET signaling. EIN2 is downstream of the ET receptors and CTR1 (Hall and Bleecker, 2003). Downstream of EIN2, the EIN3 transcription factor activates a transcription factor cascade, including the Ethylene Responsive Factor1 (ERF1) transcription factor. ERF1 regulates expression of downstream JA/ET-associated defense genes including *PDF1.2* (Lorenzo et al., 2003). Interestingly, *ANAC055* and *ANAC092* possess RAV1 binding sites in their promoter regions (Chapter 2). RAV1 is a member of the AP2/ERF transcription factor family (Kagaya et al., 1999). Collectively, this data suggests that ANAC055 and ANAC092 may be downstream of EIN2 and RAV1.

The absence of an ARR defect in *etr1-4* plants appears to contradict the idea that EIN2-dependent signaling contributes to ARR. However, *EIN2* is the only gene in which a null loss-of-function mutation results in complete ET insensitivity and decreased *PDF1.2a* expression in response to MeJA treatment, suggesting that EIN2 is important for ET and JA signaling (Alonso et al., 1999). Furthermore, expression of *PDF1.2* and *ANAC092* is reduced in *ein2* compared to *etr1* in young plants (Penninckx et al., 1996; He et al., 2005), suggesting that some JA/ET signaling is present in *etr1* (Penninckx et al., 1996). The partial JA/ET signaling in *etr1* may contribute to its ARR-competent phenotype.

EIN2 plays a role in a number of pathogen resistance responses (van Loon et al., 2006). For example, EIN2 is required for systemic signaling during the compatible interaction between Arabidopsis and CaMV (Love et al., 2005). Additionally, young *ein2* plants display enhanced resistance to virulent *Pst*, suggesting that EIN2 contributes to negative regulation of PTI in young plants (Bent et al., 1992; Chen et al., 2009). Interestingly, plants that overexpress *EIN2* do not exhibit constitutive ET signaling, but overexpressing the C-terminus of EIN2 confers a constitutive ethylene signaling phenotype, including elongated hypocotyls, only in mature Arabidopsis (after four wpg under long day conditions) (Alonso et al., 1999). This coincides with the onset of ARR observed in Arabidopsis at four wpg under long days (Rusterucci et al., 2005). This data supports the idea that EIN2 plays a role in mature ARR-competent plants.

The roles of ANAC055, ANAC092, and EIN2 in JA/ET signaling during ARR suggests that JA, ET, or JA and ET signaling contribute to ARR. To determine if JA signaling contributes to ARR we tested the ARR phenotype of *lox2*, a mutant compromised in JA accumulation and signaling (Bell et al., 1995; Spoel et al., 2003). Mature *lox2* displays wild-type resistance to *Pst*, suggesting that *lox2* exhibits no ARR defect. This is consistent with previous research that demonstrated that the *jin1-1* and *jar1-1* JA signaling mutants are also competent for ARR (Carviel et al., 2009). LOX2 is responsible for one of the first steps in JA biosynthesis: converting the fatty acid linolenic acid into 13-hydroperoxylinolenic acid (Bell and Mullet, 1993; Bell et al., 1995; Spoel et al., 2003). After JA biosynthesis, JAR1 conjugates JA to amino acids, such as isoleucine (Staswick et al., 2002; Staswick and Tiryaki, 2004). Data suggests that JA-Ile is

perceived by the F-box protein component of the ubiquitin E3 ligase SCF^{COI1} Coronatine Insensitive1 (COI1: Katsir et al., 2008; Melotto et al., 2008; Yan et al., 2009). This interaction promotes an association between COI1 and members of the Jasmonate ZIM-domain (JAZ) family (Katsir et al., 2008; Melotto et al., 2008). Several JAZ proteins are directed to the proteasome for degradation in response to JA treatment in a manner that is dependent on ubiquitination by SCF^{COI1} (Chini et al., 2007; Thines et al., 2007). JAZ proteins are thought to function as negative regulators of JA signaling (Thines et al., 2007; Yan et al., 2007) by interacting with and inhibiting a number of transcription factors, including JIN1. This is demonstrated by data that suggest that plants with a non-functional JAZ protein overexpress JIN1-regulated genes (Chini et al., 2007). JIN1 regulates expression of many JA-responsive genes, including *PDF1.2* (Berger et al., 1996; Lorenzo et al., 2004; Dombrecht et al., 2007). The wild-type ARR phenotypes of *lox2*, *jin1*, and *jar1* suggest that this pathway is not required for JA/ET signaling during ARR.

However, *lox2* and *jin1* accumulate elevated levels of SA and display increased resistance to *Pseudomonas* compared to wild-type plants (Nickstadt et al., 2004; Prithiviraj et al. 2005). SA seems to play an anti-microbial role in the intercellular space of plants exhibiting ARR (Kus et al., 2002; Cameron and Zaton, 2004). Elevated SA levels in *lox2* and *jin1*, therefore, might mask the effects of reduced JA signaling on ARR, if any, and contribute to their wild-type ARR phenotypes. Furthermore, *jin1* display wild-type expression of some JA-associated genes in response to MeJA treatment, including *LOX2*, and it accumulates wild-type levels of JA in response to JA

application and *Pseudomonas* inoculation (Berger et al., 1996; Nickstadt et al., 2004). The partial JA signaling and wild-type JA levels present in *jin1* may also contribute to its wild-type ARR phenotype. Collectively, the ARR phenotypes of the JA and ET mutants analyzed here and in previous studies (Kus et al., 2002; Carviel et al., 2009) suggest that reduced JA signaling in *jin1-1*, *jar1-1*, and *lox2*, and reduced ET signaling in *etr1-1* may not be sufficient to produce a reduced ARR response. An ARR defect is observed, however, when EIN2, a regulator of both ET and JA signaling, is absent.

3.5d JA/ET signaling during ARR

The data presented thus far suggests that some components of JA/ET signaling, including ANAC055, ANAC092, and EIN2, are required for ARR. These proteins may contribute to JA/ET signaling during ARR. To determine if JA/ET signaling is elevated in mature compared to young plants after inoculation with *Pst*, we analyzed expression of the JA/ET signaling marker *PDF1.2a*. Mature, six week-old plants exhibiting ARR express *PDF1.2a* earlier and to an elevated level compared to young, ARR-incompetent plants. It is interesting to note that the expression pattern of *PDF1.2a* is similar to the expression pattern of *ANAC092* during ARR, as both *PDF1.2a* and *ANAC092* are expressed at < 5 minutes, 12, 24, and 48 hpi with *Pst* in mature plants (6 wpg), and both are expressed earlier during ARR compared to young plants after inoculation with *Pst* (Chapter 2; this chapter; Carviel et al., 2009). ARR is also associated with decreased expression of the SA signaling marker *PR1* in mature compared to young plants after inoculation with *Pst* (Kus et al., 2002; Rusterucci et al., 2005). Therefore, ARR is associated with up-regulation of *PDF1.2a* and down-regulation of *PR1*. As a whole, these

expression patterns suggest that expression of some JA/ET-associated genes is augmented and expression of some SA-associated genes is attenuated in mature compared to young plants after inoculation with *Pst*. Furthermore, the early expression of *PDF1.2a* and *ANAC092* during ARR (Chapter 1; this chapter; Carviel et al., 2009) suggests that mature, ARR-competent Arabidopsis plants detect the presence of *Pst* and/or react to *Pst* much earlier than young ARR-incompetent plants.

When plants are infected by a pathogen, they produce different levels of SA, JA, and ET depending on the kind of pathogen they encounter (De Vos et al., 2005). This is thought to initiate the correct response to effectively combat the invading microorganism. Crosstalk between SA, JA, and ET signaling pathways during pathogen defense is complex. Much of the literature suggests that SA-dependent signaling often positively regulates defense responses to biotrophic and hemibiotrophic pathogens, and it negatively regulates defense responses to necrotrophic pathogens. JA- and ET-dependent signaling, however, often positively regulate defense responses to necrotrophic pathogens, and they negatively regulate defense against biotrophic and hemibiotrophic pathogens. This pattern is supported by gene expression data in which expression of *PR1* is elevated during infection by biotrophic pathogens, and expression of *PDF1.2* is elevated during infection by necrotrophic pathogens (Glazebrook, 2005; Koornneef and Pieterse, 2008). There are also a number of studies that illustrate a synergistic interaction between SA and JA/ET signaling (Park et al., 2001; Devadas et al., 2002; Gu et al., 2002; De Vos et al., 2006; Leon-Reyes et al., 2009). Our studies suggest that SA and JA/ET contribute to

ARR in different ways. SA seems to play an antimicrobial role in the intercellular space of ARR-exhibiting plants, while JA/ET may contribute to ARR signaling.

3.5e Proposed model for the role of ANAC055 and ANAC092 in ARR

This report investigated the role of ANAC055, ANAC92, and JA/ET signaling during ARR, and the relationship between flowering and ARR. We propose the following model for the ARR pathway. As Arabidopsis plants mature, ANAC092, and possibly LD1, positively regulate the onset of flowering and ARR competency. During ARR, mature plants initiate a swift, effective response to infection with *Pst*, including up-regulation of *PDF1.2a*. *ANAC055* and *ANAC092* are expressed in an EIN2-dependent manner. ANAC092 inhibits expression of *LOX2* and *PDF1.2a*, while ANAC055 promotes expression of *LOX2*, *PDF1.2a*, and *ICS1* (Fig 3.8).

3.6 Materials and Methods

3.6a Plant Material and Growth Conditions

The *Arabidopsis thaliana* wild-type Columbia-0 (Col-0) plants were used along with *sid2* (C. Nawrath, University of Fribourg, Switzerland), *anac055* (SALK_014331), *anac092* (SALK_090154), *lox2* (CS3748), *ein2-1* (CS3071), *ld-1* (CS3127) (Arabidopsis Biological Resource Center, Ohio State University, USA, Alonso et al., 2003), and 35S:*ANAC092* mutant plants (line 1, S-Y. Chen, Chinese Academy of Sciences, China). Seeds were surface sterilized and germinated on Murashige and Skoog medium, grown under continuous light (100 μm^{-2} s^{-1}) for seven days, then the seedlings were transferred to soil (Sunshine Mix No. 1, Sun Gro Horticulture, Bellevue, WA) moistened with 1 g L^{-1} 20-20-20 fertilizer and grown at 21 to 23°C with a light intensity of 150 to 200 μm^{-2} s^{-1}

maintaining the humidity at 85%. Short day conditions consisted of 9 h light and 15 h of darkness, while long day conditions consisted of 16 h of light and 8 h of darkness.

3.6b *In planta Pst* Growth Assays

Arabidopsis plants were inoculated with virulent *Pseudomonas syringae* pv. *tomato* (*Pst)* strain DC3000 (rifampicin and kanamycin resistant) obtained from Dr. Andrew Bent (University of Wisconsin at Madison) (Whalen et al., 1991). *Pst* was grown to mid-log phase in King's B media and kanamycin (50 μg ml^{-1}) (Sigma), shaken over-night at room temperature (22 to 25 °C), and it was then diluted to 10^6 cfu ml^{-1} in 10 mM $MgCl_2$. This *Pst* solution was pressure infiltrated into the abaxial sides of leaves using a needless 10 ml syringe, filling the intercellular space of the entire leaf. Leaves on plants without visible inflorescence stems were inoculated at each week post germination, and *in planta* bacterial growth was determined as previously described with serial dilutions plated on King's B agar plates containing rifampicin (100 μg ml^{-1}) (Sigma) and kanamycin (50 μg ml^{-1}) (Sigma) (Kus et al., 2002). ARR assays and recording of the number of inflorescence stems from newly emerged to full length were conducted at 3 to 7 wpg.

3.6c Production of *anac055anac092* Double Mutant Plants

The *anaac055anac092* double mutant plants were produced by crossing homozygote *anac055* and *anac092* mutant plants. F1 plants were screened for heterozygous T-DNA insertions in *ANAC055* and *ANAC092* and F2 plants were screened for homozygous T-DNA insertions in *ANAC055* and *ANAC092*. PCR screening employed a three-primer PCR strategy that identified individuals with wild-type,

heterozygous, and homozygous T-DNA insertions in a single step. Gene-specific primers in combination with the LBb1 primer (The Institute for Molecular Biology and Biotechnology - MOBIX: Table 3.1) were used as described on the T-DNA Express Web site (http://www.signal.salk.edu/tdna_FAQs.html) (Fig A26). RT-PCR confirmed that expression of *ANAC055* and *ANAC092* was abolished in the *anac055anac092* double mutant (Fig 2.1; Carviel et al., 2009).

3.6d Protein Sequence Alignment

A CLUSTAL W multiple alignment analysis (Thompson et al., 1994) (San Diego Super Computer, http://workbench.sdsc.edu/) was performed with the ANAC055 and ANAC092 deduced amino acid sequences with default settings.

3.6e Analysis of Gene Expression Using RT-PCR

Leaf samples were collected from Col-0, *anac055, anac092,* 35S:*ANAC092*, *lox2*, *sid2*, and *ein2-1* plants inoculated with *Pst* as well as untreated plants to analyze gene expression. Samples were collected at various time points between 0 and 48 hpi from young (3 or 4 wpg) and mature (6 wpg) plants. RNA was extracted from leaf samples using the TRIzol method (Invitrogen). DNase treatment was performed with the Ambion DNase Free system (Applied Biosystems), and cDNA was synthesized using the SuperScript III (Invitrogen) reverse transcriptase kit, according to the manufacturers' instructions. Standard PCR conditions were used with an annealing temperature of 60°C for all primers (The Institute for Molecular Biology and Biotechnology – MOBIX: Table 3.1). *ACTIN1* was used as the constitutive internal control. All gels were stained with ethidium bromide.

3.6f Cloning and Sequence Analysis of the *ANAC055* and *ANAC092* Primer Products

Due to the large size of the *NAC* gene family, it is possible that the *ANAC055* and *ANAC092* primers may amplify other *NAC* genes. To confirm that the *ANAC055* and *ANAC092* primers amplified their intended target gene, the RT-PCR products were cloned and sequenced. This confirmed that the sequences of the cloned PCR products were that of *ANAC055* and *ANAC092* (data not shown), and thus ensured that these primers were gene-specific. RNA was extracted from young (3 wpg) Col-0 leaves at 12 hpi with *Pst* with the ToTALLY RNA kit (Applied Biosystems), DNase treatment was performed with the Ambion DNase Free system (Applied Biosystems), and cDNA was synthesized using the SuperScript II (Invitrogen) reverse transcriptase kit, according to the manufacturers' instructions. The *ANAC055* and *ANAC092* primers (The Institute for Molecular Biology and Biotechnology – MOBIX: Table 3.1) were used in a standard PCR reaction with the Platinum HiFi Taq polymerase (Invitrogen) with an annealing temperature of 55°C for 35 cycles. To add an adenosine to the end of PCR products for TA cloning, Taq polymerase was added to the PCR mixture and heated to 72°C for 10 min. The PCR mixture was run on a 1% agarose gel and visualized under ultraviolet light to ensure that only one band was amplified by the primers. The PCR mixture was used to clone the PCR products using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. One Shot TOP10 Chemically Competent *Escherichia coli* (Invitrogen) cells were transformed according to the manufacturer's instructions, and plated on lysogeny broth (LB) plates supplemented with kanamycin (10 μg/ml) (Sigma)

at 37°C overnight. The plasmid DNA was extracted from an overnight *E. coli* culture in LB^{kan} media as described in Berghammer et al. (1993), and subsequently cleaned with a standard phenol-chloroform method. Plasmids containing the *ANAC055* and *ANAC092* PCR products were digested with XhoI and BamH1 (Roche), respectively, to ensure that these products were cloned into the vector, then they were sent to The Institute for Molecular Biology and Biotechnology at McMaster University for sequencing.

3.7 Acknowledgements

We thank ABRC for T-DNA insertion lines, and Dr. S-Y. Chen for providing the 35S:*ANAC092* seeds (Chinese Academy of Sciences, China). We would also like to thank Dr. Finan (McMaster University) for helpful comments on the manuscript before submission.

Chapter 4:

SA and JA signaling negatively regulate expression of *ANAC055* and *ANAC092* during ARR

4.1 Preface

All experiments contained within Chapter 4 were performed by F. Al-Daoud. As indicated, some of the results presented in this chapter are preliminary since some of the experiments were performed one time.

4.2 Abstract

Age-related resistance (ARR) refers to increased resistance to pathogens as plants age. *Arabidopsis thaliana* exhibits increased resistance to virulent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) as it ages. ARR requires salicylic acid (SA) accumulation in the intercellular space, some jasmonic acid/ethylene (JA/ET)-associated genes, and it is associated with flowering. Data presented in previous chapters suggest that two JA/ET-associated *No Apical Meristem Cup-shaped Cotyledon* (*NAC*) transcription factors, *ANAC055* and *ANAC092*, encode for regulators of JA/ET-associated gene expression (*Lipoxygenase2* and *Plant Defensin 1.2a*) during ARR in an Ethylene Insensitive2 (EIN2)-dependent pathway. Here, the role of JA signaling in ARR is investigated further by determining the ARR phenotype of the JA signaling mutants *coronatine insensitive1* (*coi1*) and *oxo-phytodienoic acid reductase3* (*opr3*). Preliminary data illustrates that these mutants exhibit enhanced ARR, suggesting that COI1 and OPR3 negatively regulate ARR. In addition, regulation of the *NAC*s was studied by characterizing expression of the *NAC*s in SA and JA signaling mutants, and in response to infection with a coronatine-deficient *Pst* strain (DC3118). The data illustrates that *ANAC055* and *ANAC092* are expressed to higher levels in JA (*lipoxygenase2* (*lox2*)) and SA (*salicylic acid induction-deficient2* (*sid2*)) accumulation-deficient mutants compared to wild-type

plants, but they are expressed to similar levels after infection with DC3118 compared to wild-type *Pst*. This suggests that SA and JA signaling negatively regulate expression of *ANAC092* and *ANAC055* during ARR, whereas coronatine does not seem to regulate *ANAC092* and *ANAC055* expression.

4.3 Introduction

There are three main phytohormones involved in plant defense: salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Bari and Jones, 2009; Grant and Jones, 2009). SA signaling mainly contributes to defense against biotrophic and hemibiotrophic pathogens, such as *Pseudomonas* bacteria, whereas JA/ET signaling usually promote defense against necrotrophic pathogens, such as some fungal species. There is significant crosstalk between SA, JA, and ET signaling during pathogen defense. In most cases JA and ET have a synergistic interaction, while the interaction between SA and JA/ET is usually characterized by an antagonistic relationship (Bari and Jones, 2009; Grant and Jones, 2009). However, there are a number of reports that demonstrate a synergistic interaction between SA and JA/ET during some pathogen defense responses (Devadas et al., 2002; De Vos et al., 2006).

JA is synthesized *in planta* by converting the fatty acid linolenic acid (LA) into 13-hydroperoxylinolenic acid. Lipoxygenase2 (LOX2) is one enzyme that is required for this conversion (Bell et al., 1995; Bell and Mullet, 1993). Then, allene oxide synthase (AOS), a member of the cytochrome P450 (CYP) family, uses 13-hydroperoxylinolenic acid to produce the JA precursor 12-oxo-phytodienoic acid (OPDA: Laudert and Weiler, 1998; von Malek et al., 2002; Park et al., 2002). OPDA is reduced by an oxo-

phytodienoic acid reductase (OPR3: Sanders et al., 2000; Stintzi and Browse, 2000), which is followed by three ß–oxidation steps to produce JA (Fig 4.1) (Feussner and Wasternack, 2002; Kazan and Manners, 2008; Turner et al., 2002). LOX2, AOS, and OPR3 are all required for JA accumulation (Bell et al., 1995; Bell and Mullet, 1993; Sanders et al., 2000; Stintzi and Browse, 2000; Park et al., 2002; Spoel et al., 2003; Stenzel et al., 2003; Raacke et al., 2006).

JA is present in a number of different forms *in planta*, including methyl jasmonate (MeJA), and it can be conjugated to some amino acids including isoleucine (Ile) (Staswick et al., 2002; Staswick and Tiryaki, 2004). JA is conjugated to amino acids by Glucocorticoid Hormone3 (GH3) proteins, such as Jasmonic Acid Resistant1 (JAR1) (Staswick et al., 2002). OPDA, JA, and JA derivatives are biologically active and regulate expression of some common target genes (McGrath et al., 2005; Sasaki-Sekimoto et al., 2005; Mueller et al., 2008; Wang et al., 2008). JA-Ile and its *Pst*-produced mimic coronatine, interact with the Coronatine Insensitive1 (COI1) component of the ubiquitin E3 ligase SCF^{COI1} (SKP1, Cullin, and F-box proteins) (Katsir et al., 2008; Melotto et al., 2008). This interaction promotes an association between COI1 and members of the Jasmonate ZIM-domain (JAZ) family (Katsir et al., 2008; Melotto et al., 2008). Several JAZ proteins are directed to the proteasome for degradation in response to JA treatment in a manner that is dependent on ubiquitination by SCF^{COI1} (Chini et al., 2007; Thines et al., 2007). JAZ proteins are thought to function as negative regulators of JA signaling (Thines et al., 2007; Yan et al., 2007) by interacting with and inhibiting a number of transcription factors, including the helix-loop-helix transcription factor

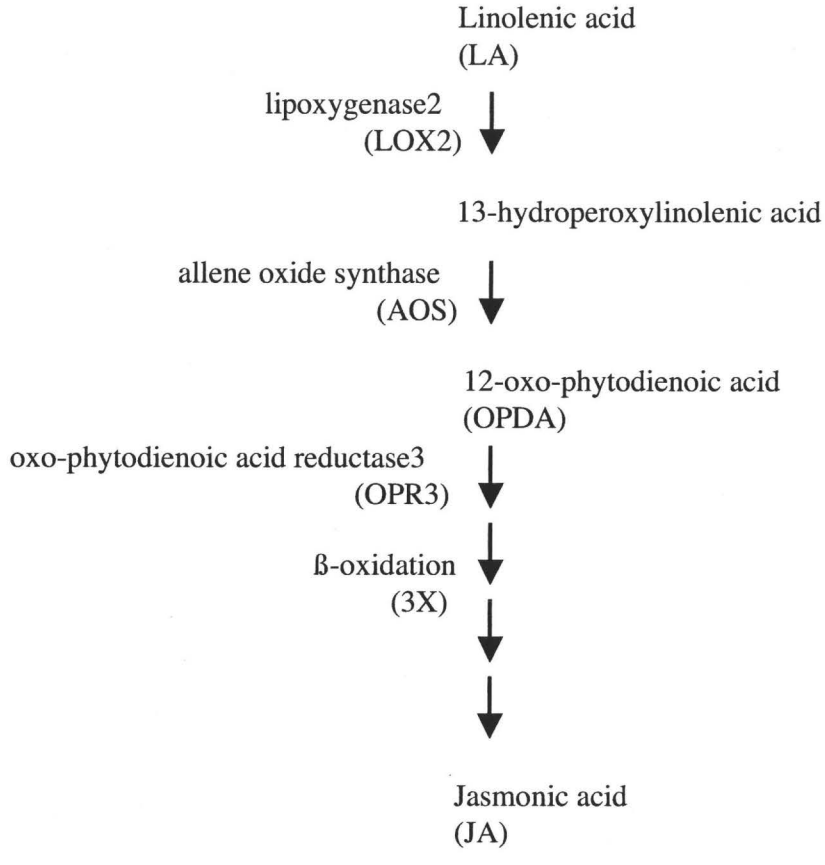


Figure 4.1. JA biosynthesis pathway. LOX2 is one of the enzymes that converts LA into 13-hydroperoxylinolenic acid. AOS then uses 13-hydroperoxylinolenic acid to produce OPDA. OPDA undergoes reduction by OPR3 and three ß-oxidation steps to form JA. Refer to text for details.

Jasmonate Insensitive1 (JIN1) (Chini et al., 2007). JIN1 regulates expression of many JA-responsive genes such as *Vegetative Storage Protein1* (*VSP1*) and the JA/ET signaling marker gene *Plant Defensin1.2* (*PDF1.2*; Fig 1.1) (Berger et al., 1996; Lorenzo et al., 2004; Dombrecht et al., 2007). This pathway contributes to defense responses against a wide variety of necrotrophic pathogens.

To overcome plant defense systems, pathogens have evolved a battery of weapons. For example, *Pseudomonas* species possess a type III secretion system (TTSS) that delivers bacterial virulence proteins into plant cells. These effector molecules act to disrupt cell metabolism and impair defense responses (Alfano and Collmer, 2004; Nomura et al., 2005). *Pseudomonas* also produces a number of phytotoxins that inhibit plant defense responses (Bender et al., 1999), including coronatine. Coronatine consists of two moieties, coronafacic acid (CFA) and coronamic acid (CMA), that mimic JA and 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene, respectively (Feys et al., 1994; Ferguson and Mitchell, 1985). Coronatine is thought to diffuse into plant cells and up-regulate JA and ET signaling thereby reducing SA signaling and promoting susceptibility to *Pseudomonas* (Ferguson and Mitchell, 1985; Zhao et al., 2003; He et al., 2004; Block et al., 2005; Brooks et al., 2005; Melotto et al., 2006; Katsir et al., 2008; Melotto et al., 2008).

Plant resistance to infection by pathogens has been observed to vary as plants age. Young Arabidopsis (*Arabidopsis thaliana*), for example, initiate a resistance response known as basal resistance or pathogen- or microbe-associated molecular pattern (PAMP)-triggered immunity (PTI) when they are infected with the virulent *Pseudomonas syringae*

pv. *tomato* DC3000 strain (*Pst*). PTI is activated when plant cell surface receptors perceive PAMPs. However, *Pst* inhibits PTI (as discussed above) and is able to successfully colonize Arabidopsis (Boller and He, 2009). As Arabidopsis ages it becomes more resistant to *Pst* (Kus et al., 2002; Rusterucci et al., 2005; Carviel et al., 2009). Increased resistance to pathogens in older plants is known as age-related resistance (ARR). There is a decrease in *Pst* levels (up to 100-fold) associated with ARR in mature (6 wpg) compared to young (3 wpg) Arabidopsis (under short days; Kus et al., 2002). SA accumulation is necessary for ARR, but it does not appear to play its usual signaling role to up-regulate *Pathogenesis Related* (*PR*) gene expression. Instead, evidence suggests that SA plays an anti-microbial role in the intercellular space of plants exhibiting ARR (Kus et al., 2002; Cameron and Zaton, 2004; Rusterucci et al., 2005; Carviel et al., 2009). Expression of the SA signaling marker *PR1* is reduced during ARR (Kus et al., 2002; Rusterucci et al., 2005), while the JA/ET signaling marker *PDF1.2a* is expressed earlier and to elevated levels in mature compared to young plants after inoculation with *Pst* (Chapter 3), suggesting that SA signaling is attenuated whereas JA/ET signaling is enhanced during ARR compared to young plants after inoculation with *Pst*.

JA/ET signaling was also implicated as being involved in ARR when expression of a number of JA/ET-associated genes was up-regulated in an ARR microarray experiment that compared gene expression in mock-inoculated mature plants versus plants that were inoculated with *Pst* (Chapter 2; Carviel et al., 2009). Two JA/ET-associated *No Apical Meristem Cup-shaped Cotyledon* (*NAC*) genes, *ANAC055* and *ANAC092*, that encode for transcription factors were up-regulated in this microarray

(Chapter 2; Carviel et al., 2009). *anac055* and *anac092* exhibit partial ARR defects, suggesting that ANAC055 and ANAC092 contribute to ARR (Chapter 2; Carviel et al., 2009). Data suggests that ANAC055 is a positive regulator of expression of some JA/ET-associated genes (*LOX2* and *PDF1.2a*) and the SA biosynthesis gene *Isochorismate Synthase1* (*ICS1*), while ANAC092 is a negative regulator of expression of some JA/ET-associated genes (*LOX2* and *PDF1.2a*) during ARR (Chapter 3). ANAC092 also appears to positively regulate the onset of ARR and flowering (Chapter 3). Furthermore, the JA/ET signaling mutant *ethylene insensitive2-1* (*ein2-1*) exhibits an ARR defect and attenuated expression of *ANAC055* and *ANAC092* during ARR, suggesting that EIN2 is a positive regulator of expression of the *NAC*s during ARR (Chapter 3). Collectively, this data suggests that JA/ET signaling contributes to ARR.

To determine if JA is required for ARR, the ARR phenotype of the *lox2* JA biosynthesis mutant was determined (Chapter 3). *lox2* plants have similar levels of JA at 48 hpi with *Pst* as untreated plants (< 10 ng g^{-1} fresh weight), whereas wild-type plants accumulate approximately 500 ng g^{-1} fresh weight of JA at 48 hpi with *Pst* (five week-old plants grown under short days; Spoel et al., 2003). *lox2* plants are competent for ARR (Chapter 3). Furthermore, the *jin1-1* and *jar1-1* JA signaling mutants are also competent for ARR (Chapter 3; Carviel et al., 2009). Collectively, this data suggests that JA/ET signaling during ARR does not require LOX2, JIN1, or JAR1.

jin1 and *lox2* may not be ideal mutants to use for studying the effect of reduced JA signaling on ARR. For example, *jin1* displays wild-type expression of some JA-associated genes, including *LOX2*, in response to MeJA treatment (Berger et al., 1996;

Nickstadt et al., 2004), suggesting that it exhibits partial JA signaling. This may contribute to the wild-type ARR phenotype of *jin1*. LOX2 is responsible for production of a number of oxylipins upstream of OPDA and JA biosynthesis (Porta and Rocha-Sosa, 2002), suggesting that *lox2* may be deficient in accumulating not only JA but some of its precursors as well. The wild-type ARR phenotype of *lox2* may be associated with lower levels of JA, some of its precursors, and other oxylipins, and may not necessarily reflect how reducing only JA levels would affect ARR.

Other JA signaling mutants may be better candidates to use for studying the role of JA signaling during ARR. For example, COI1 is thought to be required for expression of over 80% of JA-associated genes (Devoto et al., 2005; Wang et al., 2008). OPR3 is downstream of LOX2 and OPDA production, and upstream of JA biosynthesis (Fig 4.1) (Sanders et al., 2000; Stintzi and Browse, 2000), suggesting that *opr3* has decreased JA levels and wild-type OPDA levels. Wounded *opr3* plants do not accumulate JA to higher levels than untreated plants (< 1 ng g^{-1} fresh weight), whereas wounded wild-type plants accumulate up to 6 ng g^{-1} fresh weight of JA (within 6 hrs; four- to six leaf stage plants grown under short days; Stintzi et al., 2001). Characterizing ARR in *coi1* might demonstrate how reducing the majority of JA-associated gene expression would affect ARR, and characterizing the ARR phenotype of *opr3* might shed light on how lowering JA levels (without lowering OPDA levels) would impact ARR.

In this chapter, the role of JA signaling in ARR is investigated further by determining the ARR phenotype of *opr3* (JA biosynthesis mutant) and *coi1* (JA signaling mutant). Moreover, regulation of *ANAC055* and *ANAC092* expression by JA and SA

signaling, and coronatine during ARR is examined by characterizing expression of the *NAC*s in the *coi1-17* JA signaling mutant and *lox2* JA biosynthesis mutant, the *salicylic acid induction-deficient2* (*sid2*) SA accumulation-deficient mutant, and in response to inoculation with the coronatine-deficient *Pst* DC3118. In addition, regulation of expression of *ANAC055* by ANAC092 during ARR is determined by analyzing expression of *ANAC055* in the 35S:*ANAC092* background. Also, the role of a third NAC (ANAC029) whose gene was up-regulated in the ARR microarray (Chapter 2) is investigated by determining the ARR phenotype of *anac029* mutants.

4.4 Results

4.4a *coi1-17* and *opr3* exhibit an enhanced ARR

To determine the ARR phenotype of *coi1-17* and *opr3*, plants were inoculated with 10^6 colony forming units per ml (cfu ml^{-1}) of *Pst* at six weeks post germination (wpg) under short days. The level of *in planta* bacterial growth was quantified three days post inoculation. Since *coi1-17* is in the Columbia-0 (Col-0) background and *opr3* is in the Wassilewskija-2 (Ws-2) background, Col-0 and Ws-2 were also included in these ARR assays as wild-type controls. The ARR-deficient *sid2* (Col-0 background) was included as a negative control. *lox2* was also included to compare its ARR-competent phenotype with the other JA signaling mutants. Col-0 and Ws-2 plants supported low levels of *Pst* typical of plants exhibiting ARR (Col-0: 2.1 x 10^5 +/- 1.1 x 10^5 cfu per leaf disc (cfu ld^{-1}), Ws-2: 5.1 x 10^5 +/- 4.9 x 10^4 cfu ld^{-1}; Fig 4.2A). *sid2* supported 40-fold higher *Pst* levels than the wild-type (8.5 x 10^6 +/- 2.9 x 10^6 cfu ld^{-1}; $P < 0.05$ Student's T-test; Fig 4.2A). As observed in Chapter 3, *lox2* supported wild-type levels of *Pst* (2.5 x

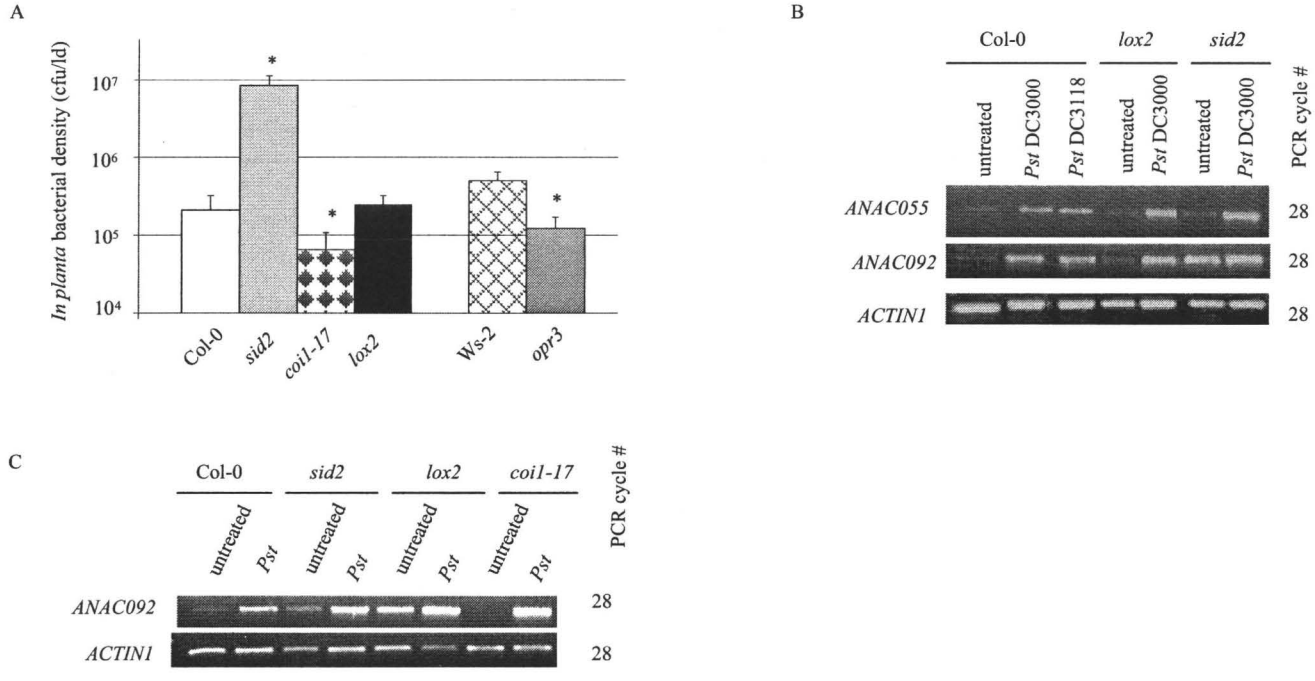


Figure 4.2. A, Preliminary data showing *in planta Pst* growth (logarithmic scale) in mature (6 wpg) Col-0, *sid2*, *coi1-17*, *lox2*, Ws-2, and *opr3*. * $P < 0.05$ Student's T-Test with each wild-type and mutant pair. For details of *Pst* quantification refer to the legend of Fig 3.1. B, *ANAC055* and *ANAC092* expression in untreated leaves (un) and at 24 hpi (10^6 cfu ml^{-1} of *Pst*) of mature (6 wpg) Col-0, *lox2*, and *sid2*. This experiment was performed three times with similar results (see Appendix A Fig A30 for replicates). *ANAC055* and *ANAC092* expression is also shown at 24 hpi with *Pst* DC3118 (10^6 cfu ml^{-1}) of mature Col-0. C, *ANAC092* expression in untreated leaves (un) and at 24 hpi (10^6 cfu ml^{-1} of *Pst*) of the same mature (6 wpg) Col-0, *sid2*, *lox2*, and *coi1-17* in part A. *ACTIN1* is included as a loading control. The number of PCR cycles is shown.

10^5 +/- 7.2 x 10^4 cfu ld^{-1}; Fig 4.2A). However, *coi1-17* supported three-fold less *Pst* than Col-0 (6.5 x 10^4 +/- 4.3 x 10^4 cfu ld^{-1}; $P < 0.05$ Student's T-test), and *opr3* supported four-fold less *Pst* than Ws-2 (1.2 x 10^5 +/- 4.9 x 10^4 cfu ld^{-1}; $P < 0.05$ Student's T-test; Fig 4.2A), suggesting that *coi1* and *opr3* exhibit an enhanced ARR. This preliminary data suggests that COI1 and OPR3 contribute to negative regulation of ARR.

4.4b Expression of *ANAC055* and *ANAC092* is elevated in mature *lox2*, *coi1*, and *sid2* after inoculation with *Pst*

ANAC055 and *ANAC092* are expressed in an EIN2-dependent manner during ARR, suggesting that they are regulated by JA/ET signaling (Chapter 3). The *NAC*s may be regulated by other components of JA signaling and by the *Pst* phytotoxin coronatine (as hypothesized by Carviel et al., 2009), which mimics JA and ET. Since SA is important for ARR (Kus et al., 2002), it might also regulate expression of *ANAC055* and *ANAC092*. To determine if JA and SA signaling and coronatine regulate expression of the *NAC*s, Reverse Transcription Polymerase Chain Reaction (RT-PCR) was utilized to observe *ANAC055* and *ANAC092* expression in mature (6 wpg) Col-0, JA accumulation-deficient *lox2*, SA accumulation-deficient *sid2*, and in response to coronatine-deficient *Pst* DC3118 (untreated and 24 hpi). Leaf samples were collected from an ARR experiment in which Col-0 displayed a modest, but significant, six-fold decrease in *Pst* levels in six week-old (2.9 x 10^6 +/- 1.5 x 10^6 cfu ld^{-1}) compared to three week-old plants (1.6 x 10^7 +/- 2.1 x 10^6 cfu ld^{-1}; $P < 0.05$ Student's T-test). *sid2* supported five-fold higher *Pst* levels than the wild-type (1.3 x 10^7 +/- 3.8 x 10^6 cfu ld^{-1}; $P < 0.05$ Student's T-test), and *lox2* supported four-fold more *Pst* than the wild-type (1.2 x 10^7 +/- 5.2 x 10^6

cfu ld^{-1}; $P < 0.05$ Student's T-test) at 6 wpg. Note that this is the only experiment (one out of four) where *lox2* exhibited an ARR defect. Therefore, expression of the *NAC*s during this unusual experiment may not reflect their expression patterns under more normal experimental conditions. However, a similar expression pattern for *ANAC092* was seen in an independent experiment (discussed below), suggesting that *NAC* expression in this experiment may be similar to their expression patterns under more normal experimental conditions.

As observed previously (Chapter 2, Chapter 3), *ANAC055* and *ANAC092* were up-regulated during ARR in Col-0, however, they were expressed to higher levels in *lox2* and *sid2* at 24 hpi (Fig 4.2B). *ANAC092* was also up-regulated in untreated *sid2* compared to the wild-type (Fig 4.2B). Furthermore, the *NAC*s were expressed to similar levels in response to inoculation with *Pst* DC3118 compared to *Pst* DC3000 (Fig 4.2B). As a whole, this data suggests that JA and SA signaling negatively regulate expression of *ANAC055* and *ANAC092*. However, this preliminary data suggests that *ANAC055* and *ANAC092* expression during ARR is not regulated by the *Pst* phytotoxin coronatine.

As COI1 is an important regulator of some JA-associated gene expression (Devoto et al., 2005; Wang et al., 2008), it might also regulate expression of the *NAC*s during ARR. To determine if COI1 regulates expression of *ANAC092*, RT-PCR was performed on samples collected from the ARR experiment described in the previous section, which included Col-0, *sid2*, *lox2*, and *coi1-17* (Fig 4.2A). Once again, *ANAC092* was up-regulated during ARR in Col-0 (Fig 4.2C), and similar to what was observed in Fig 4.2B, it was expressed to higher levels in *sid2* and *lox2* at 24 hpi (Fig 4.2C). This

supports the preliminary data in Fig 4.2B that suggested that expression of *ANAC092* was negatively regulated by JA and SA signaling during ARR. However, unlike in Fig 4.2B, *ANAC092* was up-regulated in untreated *lox2* compared to Col-0 (Fig 4.2C). *coi1-17* also had elevated levels of *ANAC092* expression compared to the wild-type at 24 hpi (Fig 4.2C). This preliminary data suggests that COI1 contributes to negative regulation of *ANAC092* expression during ARR.

4.4c *ANAC055* expression is up-regulated in 35S:*ANAC092*

anac055anac092 double mutant analysis and attenuated expression of *ANAC055* and *ANAC092* in *ein2-1* compared to the wild-type suggest that ANAC055 and ANAC092 are in the same EIN2-dependent ARR pathway (Chapter 3). As the *NAC*s code for functional transcription factors (Tran et al., 2004; He et al., 2005; Bu et al., 2008; Wang et al., 2008), they might regulate each other in this pathway. If ANAC092 regulates expression of *ANAC055,* then expression of *ANAC055* might be altered in 35S:*ANAC092*. To test this prediction, the expression of *ANAC055* was examined in mature (6 wpg) Col-0 and 35S:*ANAC092* (untreated and at 24 hpi with *Pst*: Fig 4.3). *anac055* was included as a negative control for *ANAC055* expression. Leaf samples were collected from an ARR experiment where Col-0 displayed 10-fold less *Pst* in mature (1.4 x 10^6 +/- 5.2 x 10^4 cfu ld^{-1}) compared to young plants (5 wpg: 1.3 x 10^7 +/- 1.1 x 10^6 cfu ld^{-1}; $P < 0.05$ Student's T-test). *anac055* (2.2 x 10^6 +/- 1.4 x 10^6 cfu ld^{-1}; $P = 0.22$ Student's T-test) and 35S:*ANAC092* (2.5 x 10^6 +/- 7.3 x 10^5 cfu ld^{-1}; $P < 0.05$ Student's T-test) supported two-fold higher *Pst* levels compared to the wild-type at six wpg. RT-PCR and quantitative (q)RT-PCR analyses demonstrated that *ANAC055* was expressed to

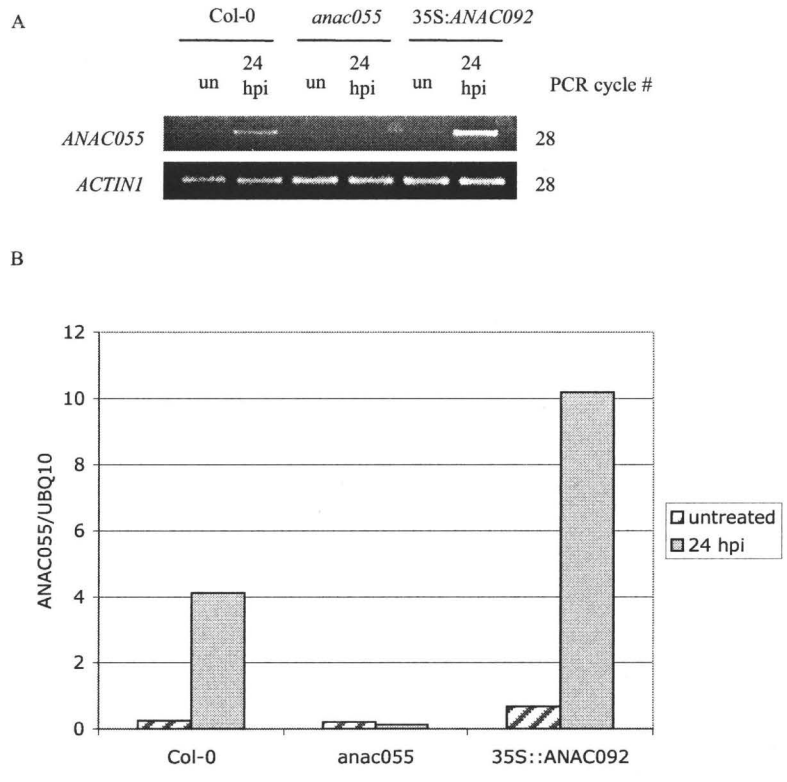


Figure 4.3. A, Preliminary RT-PCR analysis of *ANAC055* expression in leaves of mature plants (6 wpg). Samples were collected from untreated (un) Col-0, *anac055*, and 35S:*ANAC092* and at 24 hpi with 10^6 cfu/ml of *Pst*. *ACTIN1* is included as a loading control. The number of PCR cycles is shown. B, Preliminary quantitative RT-PCR analysis of *ANAC055* expression in the same samples in part A. The expression level of *ANAC055* was normalized using the expression level of *UBQ10* as an internal control.

a higher level (> 2.5-fold) in 35S:*ANAC092* compared to Col-0 (Fig 4.3A,B). This preliminary data suggests that ANAC092 is a positive regulator of *ANAC055* expression during ARR.

4.4d *anac029* displays wild-type ARR

The ARR microarray performed by Carviel et al. (2009) identified a third *NAC* gene that is expressed during ARR, *ANAC029* (1.8-fold increased expression in six week-old plants inoculated with *Pst* compared to mock-inoculated plants; http://bar.utoronto.ca/affydb/cgi-bin/affy_db_proj_browser.cgi). This suggests that ANAC029 may contribute to ARR. To determine if ANAC029 is required for ARR, mature *anac029* plants (6 wpg) were included in an ARR assay with Col-0 and the ARR-defective *sid2* (Fig 4.4). Col-0 supported low *Pst* levels (6.0×10^5 +/- 3.1×10^5 cfu ld^{-1}), confirming that they exhibited ARR, whereas *sid2* supported 25-fold more *Pst* (1.2×10^7 +/- 2.2×10^6 cfu ld^{-1}; $P < 0.05$ Student's T-test). *anac029* supported similar levels of *Pst* as Col-0 (5.1×10^5 +/- 1.7×10^5 cfu ld^{-1}), suggesting that *anac029* displays wild-type ARR. Therefore, this preliminary data suggests that ANAC029 is not required for ARR.

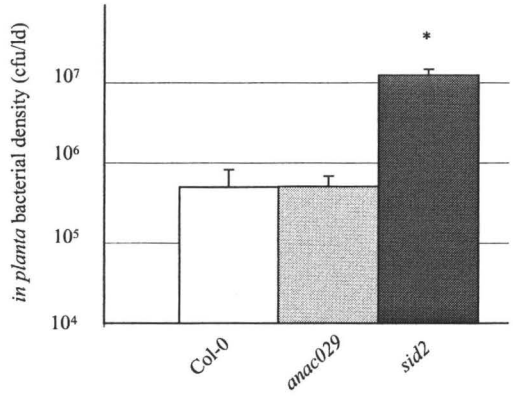


Figure 4.4. Preliminary data showing *in planta Pst* growth (logarithmic scale) in mature (6 wpg) Col-0, *anac029*, and *sid2* plants. * $P < 0.05$ student's T-Test with each wild-type and mutant pair. For details of *Pst* quantification refer to the legend of Fig 3.1.

4.5 Discussion

4.5a SA and JA signaling contribute to a negative feedback loop in the NAC ARR pathway

A preliminary experiment illustrated that *coi1-17* and *opr3* exhibit an enhanced ARR response compared to the wild-type, suggesting that COI1 and OPR3 are involved in negatively regulating ARR. The idea that some components of JA signaling negatively regulate ARR is supported by data that shows that expression of *ANAC055* and *ANAC092* is up-regulated in mature *lox2*, and preliminary data that suggests that expression of *ANAC092* is up-regulated in mature *coi1-17* compared to mature wild-type plants after inoculation with *Pst*. This suggests that JA signaling negatively regulates expression of the *NAC*s during ARR. Interestingly, *ANAC055* and *ANAC092* expression is also up-regulated in *sid2*, suggesting that some components of SA signaling are also involved in negatively regulating expression of the *NAC*s during ARR. Moreover, data from Chapter 3 suggest that *ANAC055* and *ANAC092* are expressed in an EIN2-dependent manner. As a whole, this suggests that EIN2-dependent JA/ET signaling positively regulates expression of the *NAC*s, whereas JA and SA signaling are involved in negatively regulating their expression during ARR. Also, preliminary data suggests that *ANAC055* and *ANAC092* expression is not affected when mature Arabidopsis is inoculated with coronatine-deficient *Pst* compared to wild-type *Pst*, suggesting that expression of the *NAC*s is not regulated by coronatine. Previously, expression of *ANAC055* and *ANAC092* was observed to be induced by *Pst* type III effectors in young plants (Truman et al., 2006;

Torres-Zabala et al., 2007). Therefore, *Pst* type III effectors may also regulate expression of *ANAC055* and *ANAC092* during ARR.

When the data presented in this chapter is considered with previous observations that suggest that ANAC055 positively regulates expression of *LOX2* and *ICS1*, and ANAC092 negatively regulates expression of *LOX2* during ARR (Chapter 3), it indicates the possibility of a negative feedback loop in this pathway. The NACs appear to regulate *LOX2* and *ICS1* expression, and the end products of the LOX2 and ICS1 pathways, i.e. JA and SA, respectively, seem to induce signaling pathways that negatively regulate expression of the *NAC*s thus creating a feedback loop (Fig 4.5). This model might explain why JA signaling mutants and JA biosynthesis mutants do not exhibit an ARR defect. It predicts that the elimination of the negative feedback regulation by JA signaling on the NAC pathway may increase expression of the *NAC*s in these plants, as it does in *lox2* and *coi1-17*. Increased *ANAC055* expression might result in increased expression of *ICS1* and *LOX2*, while increased *ANAC092* expression might lead to a decrease in *LOX2* expression. One outcome of this signaling pathway might be increased expression of *ICS1* and thus increased SA levels in JA mutants. This prediction is supported by data that shows that *lox2*, *jin1*, and *coi1* accumulate elevated SA levels compared to wild-type plants after infection by pathogens (Kloek et al., 2001; Nickstadt et al., 2004; Prithiviraj et al. 2005). Therefore, *lox2*, *jin1*, and *coi1* may accumulate more SA than wild-type plants during ARR. The hyper-accumulation of SA in these mutants might compensate for the effects of decreased JA levels on ARR, if any, and contribute to the

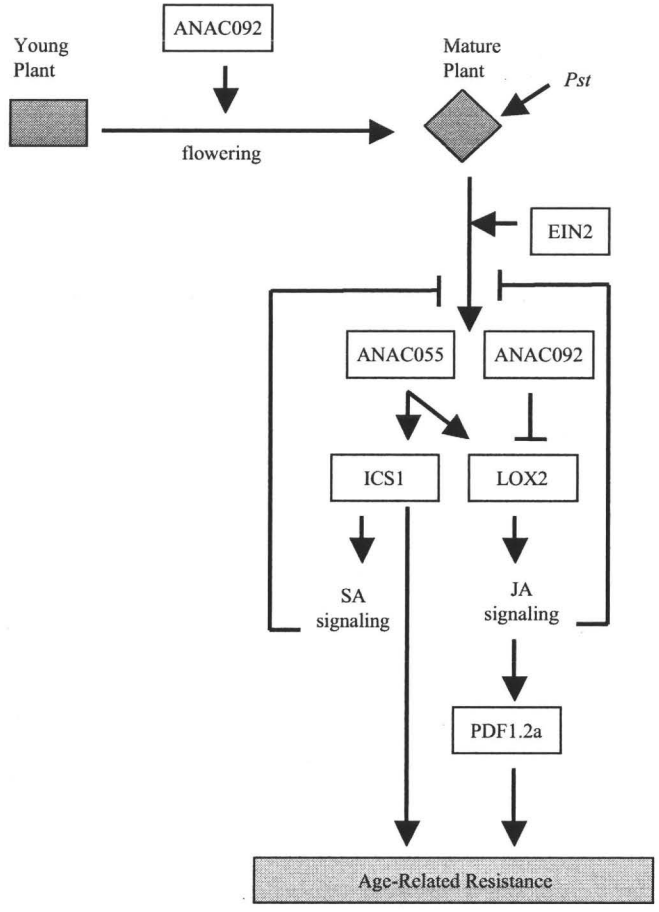


Figure 4.5. Proposed model for the role of ANAC055 and ANAC092 in the ARR pathway. As Arabidopsis grows, ANAC092 positively regulates the transition to flowering and the onset of ARR competency. After *Pst* inoculation, ARR-competent Arabidopsis up-regulates *ANAC055* and *ANAC092* expression in an EIN2-dependent manner. ANAC055 positively regulates *ICS1*, *LOX2*, and *PDF1.2a* expression, while ANAC092 negatively regulates expression of *LOX2* and *PDF1.2a*. JA and SA signaling negatively regulate expression of *ANAC055* and *ANAC092* in a feedback loop. This culminates in ARR.

wild-type ARR in *lox2* and *jin1* (Chapter 3; Carviel et al., 2009) and the enhanced ARR exhibited by *coi1* (this chapter).

The data presented thus far suggest that expression of *ANAC055* and *ANAC092* is at least partly co-regulated in the same ARR pathway, such that it is positively regulated by EIN2 and negatively regulated by JA and SA signaling (Chapter 3; this chapter). To determine if ANAC092 regulates *ANAC055* in this pathway, we observed the expression of *ANAC055* in 35S:*ANAC092*. Preliminary data suggests that *ANAC055* expression is up-regulated in 35S:*ANAC092*. ANAC092 seems to positively regulate *ANAC055*, while negatively regulating expression of other JA/ET-associated genes during ARR, including *LOX2* and *PDF1.2a* (Chapter 3). ANAC092 could be a direct regulator of *ANAC055* expression, or according to the ARR model proposed above (Fig 4.5) it might regulate expression of *ANAC055* in an indirect manner. 35S:*ANAC092* displays decreased *LOX2* and *PDF1.2a* expression in association with its ARR defect, suggesting that 35S:*ANAC092* has attenuated JA/ET signaling (Chapter 3). Since *ANAC055* expression seems to be negatively regulated by JA signaling, the reduced JA/ET signaling in 35S:*ANAC092* might result in alleviation of some negative regulation of *ANAC055*, thus leading to increased *ANAC055* expression in 35S:*ANAC092*. Therefore, there are at least two possible explanations for the increased expression of *ANAC055* in 35S:*ANAC092*. First, ANAC092 could be a direct positive regulator of *ANAC055* and negative regulator of other JA/ET-associated genes (*LOX2* and *PDF1.2a*). Second, ANAC092 may derepress expression of *ANAC055* in an indirect manner by inhibiting JA/ET signaling which negatively regulates *ANAC055*.

4.5b ANAC029 is not required for ARR

The gene product of the third *NAC* gene identified in the ARR microarray, *ANAC029* (Chapter 2; Carviel et al., 2009) does not seem to be required for ARR, as mature *anac029* supports similar *Pst* levels as the wild-type in a preliminary experiment. Alternatively, ANAC029 may contribute to ARR, but the lack of an ARR defect in *anac029* may be a result of redundancy with other NACs. As stated in Chapter 3, *ANAC029* is expressed in an EIN2-dependent manner in young, four to five week-old Arabidopsis, after inoculation with *Psm*, similar to *ANAC055* and *ANAC092* (Wang et al., 2008). MeJA treatment up-regulates *ANAC055* and *ANAC029* (McGrath et al., 2005), and ANAC029 and ANAC092 regulate leaf senescence (Guo and Gan, 2006; Kim et al., 2009). Also, the putative amino acid sequence of ANAC029 is 37% identical to ANAC055 and 36% identical to ANAC092 (Chapter 3). Taken together, this data suggests that *ANAC029*, *ANAC055*, and *ANAC092* are co-regulated, and ANAC029 may be partially redundant with ANAC055 or ANAC092 during ARR.

4.6 Materials and Methods

4.6a Plant Growth Conditions

The *Arabidopsis thaliana* wild-type Columbia (Col-0) and Wassilewskija-2 (Ws-2) plants were used along with *sid2* (C. Nawrath, University of Fribourg, Switzerland), *anac055* (SALK_014331), *anac029* (SALK_005010C), *lox2* (CS3748) (Arabidopsis Biological Resource Center, Ohio State University, USA, Alonso et al., 2003), *coi1-17* (B. Kunkel, Washington University), *opr3* (B. Kunkel, Washington University), and 35S:*ANAC092* plants (line 1, S-Y. Chen, Chinese Academy of Sciences, China). Seeds

were surface sterilized and germinated on Murashige and Skoog medium, grown under continuous light (100 $\mu m^{-2}s^{-1}$) for 7 days, then the seedlings were transferred to soil (Sunshine Mix No. 1, Sun Gro Horticulture, Bellevue, WA) moistened with 1 g L^{-1} 20-20-20 fertilizer and grown at 21 to 23 °C with a light intensity of 150 to 200 $\mu m^{-2}s^{-1}$ maintaining the humidity at 85% under short days (9 h light and 15 h of darkness).

4.6b Inoculation with *Pst*

Arabidopsis plants were inoculated with virulent *Pseudomonas syringae* pv. *tomato* (*Pst)* strain DC3000 (rifampicin and kanamycin resistant) obtained from Dr. Andrew Bent (University of Wisconsin at Madison) (Whalen et al., 1991), and *Pst* DC3118 obtained from Dr. D.A. Cuppels (Agriculture and Agri-Food Canada, London, Ontario) (Ma et al., 1991). *Pst* was grown to mid-log phase in King's B media and kanamycin (50 $\mu g\ ml^{-1}$) (Sigma), shaken overnight at room temperature (22 to 25 °C), then diluted to 10^6 colony forming units, (cfu ml^{-1}) in 10 mM $MgCl_2$, and pressure infiltrated into the abaxial sides of 8-10 leaves per plant using a needless 10 ml syringe, filling the intercellular space of the entire leaf. Leaves on plants without visible inflorescence stems were inoculated, and *in planta* bacterial growth was determined as previously described with serial dilutions plated on King's B agar plates containing rifampicin (100 $\mu g\ ml^{-1}$) (Sigma) and kanamycin (50 $\mu g\ ml^{-1}$) (Sigma) (Kus et al., 2002).

4.6c RT-PCR

Leaf samples were collected from Col-0, *anac055,* 35S:*ANAC092*, *lox2*, *sid2*, and *coi1-17* plants inoculated with *Pst* as well as untreated plants to analyze gene expression. Samples were collected from untreated plants and at 24 hours post inoculation (hpi) from

mature (6 wpg) plants. RNA was extracted from leaf samples using the TRIzol method (Invitrogen), DNase treatment was performed with the Ambion DNase Free system (Applied Biosystems), and cDNA was synthesized using the SuperScript III (Invitrogen) reverse transcriptase kit, according to the manufacturer's instructions. Standard PCR conditions were used with an annealing temperature of 60 °C for all primers (The Institute for Molecular Biology and Biotechnology - MOBIX; Table 4.1). *ACTIN1* was used as the constitutive internal control. All gels were stained with ethidium bromide.

4.6d Quantitative RT-PCR

The Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) kit was used according to the manufacturer's instructions with 96-well Optical Reaction Plates with Barcode (Applied Biosystems) and MicroAmp Opitcal Adhesive Film (Applied Biosystems). Standard qPCR conditions were used with an annealing temperature of 60 °C and melting curve analysis was performed to ensure that a single product was amplified using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) and analyzed using the SDS 2.2 software (Applied Biosystems) according to the manufacturer's directions. A logarithmic florescence signal (ΔR_n) versus cycle number graph was constructed between cycles 0 to 40. All amplification plots were analyzed with an R_n threshold of 0.20 to 0.26 to obtain the threshold cycle (C_T). A C_T versus quantity graph was constructed using the template dilution samples in order to calculate the efficiency rate (E) for each set of primers [$(1+E)=10^{(-1/slope)}$; Pfaffl, 2001]. C_T versus quantity graphs with an E of 85 to110% and an R2 value of at least 0.95 were

Table 4.1. Forward and reverse sequences of primers used in RT-PCR and qRT-PCR.

Gene name and AGI number	Primer sequences 5' → 3'
RT-PCR *ANAC055*	F[a] ATGGGTCTCCAAGAGCTTGA
(At3g15500)	R[b] TCAAATAAACCCGAACCCAC
RT-PCR *ANAC092*	F ATGGATTACGAGGCATCAAG
(At5g39610)	R TCAGAAATTCCAAACGCAAT
RT-PCR *ACTIN1*	F GGCGATGAAGCTCAATCCAAACG
(At2g37620)	R GGTCACGACCAGCAAGATCAAGACG
qRT-PCR *ANAC055*	F CGTGTCAGATTAGTTAATTAATATAGCG
(At3g15500)	R CGAATATACAATACAAGTCGTAGAACC
qRT-PCR *ANAC092*	F GAATTTCTGAGTTGTATAAGTTATGTTG
(At5g39610)	R GTAACAAGAATATTCATTCACACACACG
qRT-PCR *UBQ10*	F GGCCTTGTATAATCCCTGATGAATAAG
(At4g05320)	R AAAGAGATAACAGGAACGGAAACATAGT

[a] forward primer sequence
[b] reverse primer sequence

used to calculate the absolute transcript level of the unknown samples. Absolute transcript quantity was normalized with the absolute transcript quantity of *UBQ10*.

4.7 Acknowledgments

We thank ABRC for T-DNA insertion lines, Dr. S-Y. Chen for providing the 35S:*ANAC092* seeds (Chinese Academy of Sciences, China), Dr. D.A. Cuppels (Agriculture and Agri-Food Canada, London, Ontario) for providing *Pst* DC3118, and Dr. B. Kunkel (Washington University) for providing *coi1-17* and *opr3* seeds.

Chapter 5:

Young and mature plants have similar levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA after inoculation with *Pst*

5.1 Preface

Ms. L. Zhenjiu and Dr. L. Hicks at the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center (St. Louise, Missouri, USA), and the staff at the Plant Biotechnology Institute (Saskatoon, Saskatchewan) performed the phytohormone quantification. The material and methods section for the phytohormone analyses was kindly provided by Dr. L. Hicks (Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center, St. Louise, Missouri, USA). ARR experiments, plant sample collection, RT-PCR, and phytohormone data analyses were performed by F. Al-Daoud.

5.2 Abstract

Mature Arabidopsis (*Arabidopsis thaliana*) exhibits a developmentally-regulated resistance response to virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) known as age-related resistance (ARR). ARR requires intercellular salicylic acid (SA) accumulation, and it is associated with flowering. Expression of some JA/ET-related genes is up-regulated during ARR, and some JA/ET-associated gene products contribute to ARR. Data from previous chapters suggests that two No Apical Meristem Cup-shaped Cotyledons (NAC) transcription factors, ANAC055 and ANAC092, play non-redundant roles in an Ethylene Insensitive2 (EIN2)-dependent pathway. It appears that ANAC055 is a positive regulator of expression of some JA/ET-related genes and the SA biosynthesis gene *Isochorismate Synthase1* (*ICS1*), whereas ANAC092 is a positive regulator of the onset of ARR and flowering, and a negative regulator of some JA/ET-associated gene expression during ARR. To determine if ANAC055 and ANAC092 regulate

phytohormone accumulation during ARR and to investigate if the increased JA/ET signaling observed during ARR is associated with elevated phytohormone accumulation, the levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA were measured. Leaves from young and mature plants have similar levels of these phytohormones after inoculation with *Pst*. Furthermore, preliminary data suggests that the ARR defects of *anac055*, *anac092*, and 35S:*ANAC092* are associated with wild-type phytohormone levels.

5.3 Introduction

Plants have evolved a number of strategies to combat infection by pathogens. One of the first responses of plants to pathogen attack includes PAMP (pathogen-associated molecular pattern)-triggered immunity (PTI). PAMPs are general features common to a variety of microbes. For example, a number of bacteria possess similar membrane lipopolysaccharides and flagella proteins. Plants utilize pattern recognition receptors (PRR) to recognize PAMPs and initiate PTI signaling. This results in a number of local physiological, biochemical and molecular changes in the plant designed to slow the spread of the infection (Boller and Felix, 2009; Boller and He, 2009; Nicaise et al., 2009).

The flagellin protein that constitutes the flagellum of *Pseudomnas syringae* pv. *tomato* (*Pst*) is recognized by the Flagellin Sensing 2 (FLS2) leucine-rich repeat receptor kinase (LRR-RK) of Arabidopsis (*Arabidopsis thaliana*) (Gomez-Gomez et al., 2000). More specifically, FLS2 recognizes a 22 amino acid-long peptide at the N-terminus of flagellin called flg22 (Gomez-Gomez et al., 2000). Other Arabidopsis PRRs include the elongation factor-Tu (EF-Tu) receptor (EFR) that recognizes elf18, an 18 amino acid-

long peptide at the N-terminus of EF-Tu of *Pst* (Kunze et al., 2004; Zipfel et al., 2006). Downstream of FLS2 and EFR is the LRR receptor-like kinase (LRR-RLK) Brassinosteroid-associated Kinase 1 (BAK1: Chinchilla et al., 2007; Heese et al., 2007). BAK1 binds FLS2 and subsequently initiates PTI signaling (Chinchilla et al., 2007; Heese et al., 2007).

To overcome plant defense systems, pathogens have evolved a battery of weapons to inhibit plant immunity. For example, *Pseudomonas* species possess a type III secretion system (TTSS) that delivers virulence proteins into host cells. These effector molecules are thought to disrupt cell metabolism and impair defense responses (Alfano and Collmer, 2004; Nomura et al., 2005). *Pseudomonas* also produces a number of phytotoxins that impair plant defense responses (Bender et al., 1999), including coronatine. Many of these effectors and phytotoxins negatively regulate plant defenses by promoting accumulation of phytohormones that are involved in negative regulation of immune responses to *Pseudomonas*, including jasmonic acid (JA), auxin/indole-3-acetic acid (Aux/IAA), and abscisic acid (ABA) (Bari and Jones, 2009; Grant and Jones, 2009).

Plant susceptibility to pathogen attack varies with age (Whalen, 2005; Develey-Riviere, 2007). As Arabidopsis matures it becomes more resistant to virulent *Pst* (*Pst*) (Kus et al., 2002; Rusterucci et al., 2005; Carviel et al., 2009). Increased resistance to pathogens with age is known as age-related resistance (ARR). ARR in mature, six week-old plants is associated with decreased expression of the salicylic acid (SA) signaling-associated defense gene *Pathogenesis Related1* (*PR1*) (Kus et al., 2002; Rusterucci et al., 2005) and early and increased expression of the jasmonic acid/ethylene (JA/ET) signaling

marker *Plant Defensin 1.2a* (*PDF1.2a*) compared to young plants (at 3 weeks post germination, wpg) after inoculation with *Pst* (Chapter 3). SA may not play a signaling role during ARR, however, intercellular accumulation of SA, which is associated with anti-microbial activity, is important for ARR (Kus et al., 2002; Cameron and Zaton, 2004; Rusterucci et al., 2005; Carviel et al., 2009). Also, a number of JA/ET-related genes encode proteins that contribute to ARR, including two No Apical Meristem Cup-shaped Cotyledon (NAC) transcription factors, ANAC055 and ANAC092 (Chapter 2; Chapter 3; Carviel et al., 2009). However, some JA-associated genes, including *Jasmonate Insensitive1* (*JIN1*), *Jasmonic Acid Resistant1* (*JAR1*), *Lipoxygenase2* (*LOX2*), *Oxo-phytodienoic Acid Reductase3* (*OPR3*), and *Coronatine Insensitive1* (*COI1*) encode for proteins that are not required for ARR (Chapter 3; Chapter 4: Carviel et al., 2009).

Previous reports found that young plants (3 wpg) express *PR1* to higher levels at three dpi (days post inoculation) compared to one dpi with *Pst*, whereas mature plants (6 wpg) express *PR1* to lower levels at three dpi compare to one dpi. *PR1* seem to be expressed less during late stages of ARR (after 24 hpi) compared to young plants after inoculation with *Pst* (Kus et al., 2002; and Rusterucci et al., 2005). However, the defense gene *PDF1.2a* is expressed to elevated levels during early (before 24 hpi) and late stages (after 24 hpi) of ARR in mature plants compared to young plants after inoculation with *Pst* (Chapter 3). In a similar fashion to *PDF1.2a*, *PR1* might also be expressed to elevated levels during early stages of ARR. In this chapter, expression of *PR1* is explored during early (before 24 hpi) and late stages (after 24 hpi) of ARR compared to young plants after

inoculation with *Pst*. Also, to determine if expression of JA/ET-related genes during ARR is associated with elevated phytohormone accumulation, the phytohormone profile (JA, the JA precursor 12-oxo-phytodienoic acid (OPDA), JA-isoleucine (JA-Ile), SA, Aux/IAA, IAA-aspartic acid (IAA-Asp), and ABA) of ARR-incompetent plants is compared with plants exhibiting ARR. Furthermore, the phytohormone profile of mature *anac055*, *anac092*, and 35S:*ANAC092* is compared to wild-type plants to investigate if the ARR defects of these plants are associated with altered phytohormone levels.

5.4 Results

5.4a *PR1* expression is elevated during early stages of infection with *Pst* in mature compared to young plants

Expression of *PDF1.2a* is elevated during early (before 24 hpi) and late stages (after 24 hpi) of ARR in mature plants compared to young plants after inoculation with *Pst* (Chapter 3), suggesting that some defense genes are expressed early during ARR. Expression of *PR1*, however, is lower in mature compared to young plants after 24 hpi (Kus et al., 2002; Rusterucci et al., 2005). As *PR1* may be expressed earlier than 24 hpi during ARR, *PR1* expression was determined in leaves collected from young (4 wpg) and mature (6 wpg) wild-type Columbia-0 (Col-0) (untreated, 5 min, 12, 24, and 48 hpi with 10^6 cfu ml^{-1} of *Pst*). During ARR experiments plants are grown under short day conditions (9 hr photoperiod), and *in planta Pst* density is determined three days post inoculation (dpi). In this experiment, four week-old Col-0 supported high levels of *Pst* typical of ARR-incompetent plants (2.7×10^6 +/- 1.1×10^6 cfu ld^{-1}), and six week-old Col-0 supported low *Pst* levels typical of plants exhibiting ARR (9.2×10^4 +/- 5.1×10^4

cfu ld^{-1}). There was a 30-fold decrease in *Pst* levels observed in mature compared to young Col-0 (Student's t-test $P < 0.05$). In young plants, *PR1* was expressed to low levels at 5 min and 12 hpi, followed by elevated expression at 24 and 48 hpi (Fig 5.1). In plants exhibiting ARR, however, *PR1* was expressed to a higher level at 5 min and 12 hpi, and this was followed by a decrease in expression at 24 and 48 hpi (Fig 5.1). Overall, *PR1* was expressed to higher levels during early stages of ARR (at 5 min and 12 hpi) and it was expressed to lower levels during later stages of ARR (after 24 hpi), compared to young plants after inoculation with *Pst*. *PR1* expression after 24 hpi in young and mature plants was similar to the previously reported data, such that *PR1* expression was reduced in mature compared to young plants after 24 hpi (Kus et al., 2002; Rusterucci et al., 2005). However, these earlier reports did not monitor *PR1* expression at the 5 min and 12 hpi time points presented here. Taken together, this preliminary data and observations made in previous reports (Kus et al., 2002; Rusterucci et al., 2005) suggest that *PR1* is expressed to higher levels before 24 hpi with *Pst* in mature compared to young plants, whereas it is expressed to lower levels in mature compared to young plants after 24 hpi.

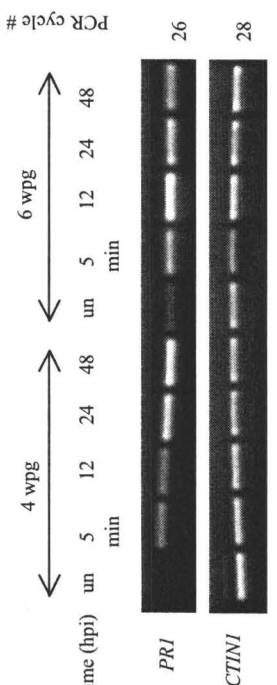


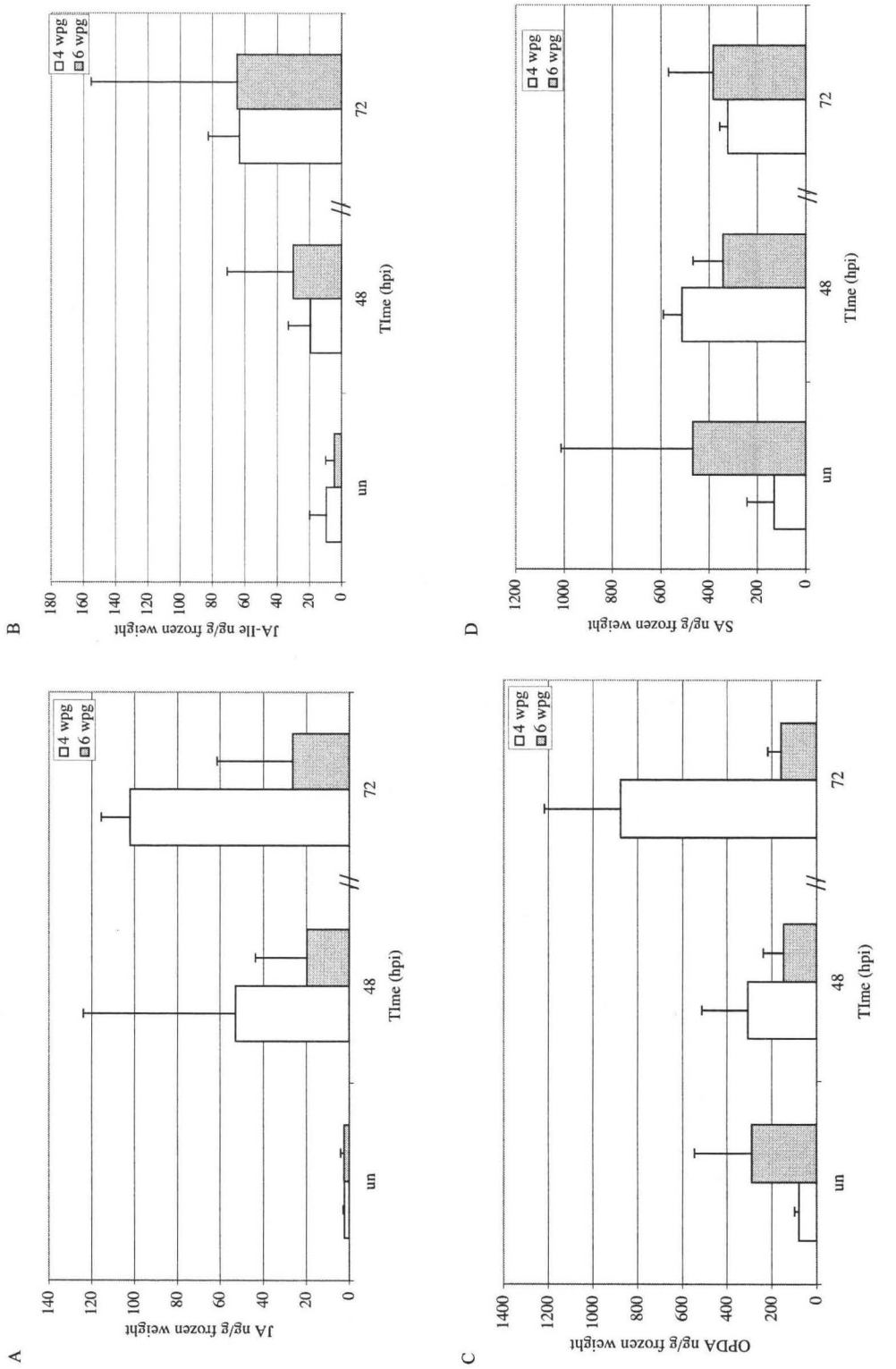
Figure 5.1. Preliminary results showing *PR1* expression in young and mature plants. Leaf samples were collected from untreated (un) Col-0 leaves and at 5 min, 12, 24, and 48 hpi with 10^6 cfu ml^{-1} of *Pst. ACTIN1* is included as a loading control. The number of PCR cycles is shown.

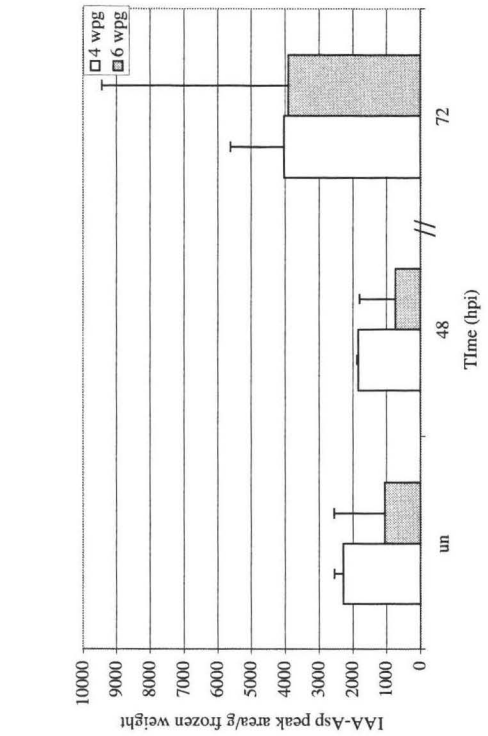
5.4b Analysis of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA levels in young and mature plants after inoculation with *Pst*

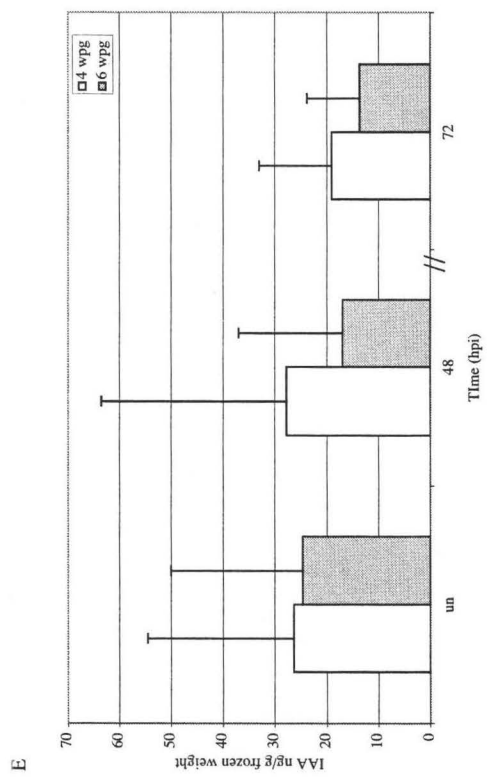
There is increased expression of some JA/ET-related genes in mature compared to young plants after inoculation with *Pst*, suggesting that some JA/ET signaling is up-regulated during ARR (Chapter 2; Chapter 3; Carviel et al., 2009). This may be indicative of greater JA accumulation in mature compared to younger plants after inoculation with *Pst*. To test this possibility, levels of free JA, JA conjugated to isoleucine (JA-Ile), and the JA precursor 12-oxo-phytodienoic acid (OPDA) were measured in leaves collected from young (4 wpg) and mature (6 wpg) Col-0 (untreated, 48, and 72 hpi). All phytohormone quantification referred to herein was performed by the Donald Danforth Plant Science Center (St. Louise, USA) unless noted otherwise. Samples were collected from two independent experiments where young, four week-old Col-0 supported high *Pst* levels ($> 10^6$ cfu ld^{-1}), and mature, six week-old Col-0 supported low *Pst* levels ($< 5 \times 10^5$ +/- cfu ld^{-1}). The average (n=2 +/- standard deviation) of the two experiments is presented (Fig 5.2). There was large variation in the data from different experiments as illustrated by the large standard deviation values.

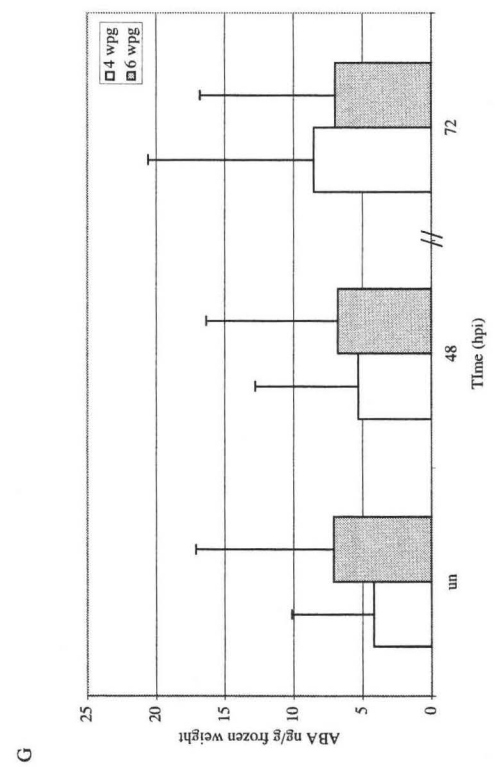
Little JA was detected in untreated young (2.3 +/- 0.5 ng g^{-1} frozen weight) and mature plants (2.3 +/- 1.6 ng g^{-1} frozen weight: Fig 5.2A). JA levels were elevated in young plants at 48 hpi (53 +/- 71 ng g^{-1} frozen weight) and 72 hpi (102 +/- 14 ng g^{-1} frozen weight). JA levels were also elevated in mature plants at 48 hpi (19.8 +/- 24 ng g^{-1} frozen weight) and 72 hpi (27 +/- 35 ng g^{-1} frozen weight). Overall, there was no

Figure 5.2. A) JA, B) JA-Ile, C) OPDA, D) free SA, E) IAA, F) IAA-Asp, and G) ABA levels in leaves of young (4 wpg) and mature (6 wpg) Col-0. Untreated (un), 48, and 72 hpi (10^6 cfu ml^{-1} of *Pst*) samples were analyzed. Each data point is the average of one replicate from two independent experiments (n=2) +/- SD (see Appendix A Fig A44, Fig A45, Fig A46, Fig A47, Fig A48, Fig A49, Fig A50, and Fig A51 for replicates).


A
JA ng/g frozen weight
0
20
40
60
80
100
120
140
4 wpg
6 wpg
un
48
72
TIme (hpi)
B
JA-Ile ng/g frozen weight
0
20
40
60
80
100
120
140
160
180
4 wpg
6 wpg
un
48
72
TIme (hpi)
C
OPDA ng/g frozen weight
0
200
400
600
800
1000
1200
1400
4 wpg
6 wpg
un
48
72
TIme (hpi)
D
SA ng/g frozen weight
0
200
400
600
800
1000
1200
4 wpg
6 wpg
un
48
72
TIme (hpi)


F
IAA-Asp peak area/g frozen weight
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
un
48
72
TIme (hpi)
4 wpg
6 wpg


E
IAA ng/g frozen weight
70
60
50
40
30
20
10
0
un
48
72
TIme (hpi)
4 wpg
6 wpg


G
ABA ng/g frozen weight
25
20
15
10
5
0
un
48
72
TIme (hpi)
4 wpg
6 wpg

significant difference in JA accumulation in young and mature plants after inoculation with *Pst*. Untreated young plants (9.4 +/- 10 ng g^{-1} frozen weight) had similar levels of JA-Ile compared to mature plants (4.5 +/- 5.1 ng g^{-1} frozen weight; Fig 5.2B). JA-Ile levels increased in young plants at 48 hpi (19 +/- 14 ng g^{-1} frozen weight), and at 72 hpi (63 +/- 19 ng g^{-1} frozen weight). Mature plants accumulated similar levels of JA-Ile as young plants after inoculation with *Pst* (48 hpi, 30 +/- 41 ng g^{-1} frozen weight; 72 hpi, 65 +/- 91 ng g^{-1} frozen weight). Untreated young plants (79 +/- 20 ng g^{-1} frozen weight; Fig 5.2C) had similar levels of OPDA compared to mature plants (290 +/- 257 ng g^{-1} frozen weight). In young plants, OPDA levels were elevated at 48 hpi (308 +/- 206 ng g^{-1} frozen weight) and 72 hpi (876 +/- 342 ng g^{-1} frozen weight) compared to untreated leaves. No significant increase in OPDA levels was observed in mature plants in response to infection with *Pst* at 48 or 72 hpi compared to untreated plants (48 hpi, 146 +/- 92 ng g^{-1} frozen weight; 72 hpi, 159 +/- 59 ng g^{-1} frozen weight). However, there was no significant difference in OPDA levels in young and mature plants after inoculation with *Pst*. Therefore, the data suggests that, contrary to what was expected, JA, JA-Ile, and OPDA accumulated to similar levels during ARR compared to young plants at 48 and 72 hpi with *Pst*.

To obtain a more complete phytohormone profile of young and mature plants after infection with *Pst*, levels of free SA, IAA, IAA conjugated to aspartic acid (IAA-Asp), and ABA were measured in the same samples described above (untreated, 48, and 72 hpi). Young plants had little SA in untreated leaves (131 +/- 111 ng g^{-1} frozen weight; Fig 5.2D). SA levels rose at 48 hpi (513 +/- 77 ng g^{-1} frozen weight) and they remained

high at 72 hpi (324 +/- 33 ng g^{-1} frozen weight). Mature plants accumulated similar levels of SA compared to young plants at 48 and 72 hpi. IAA levels were similar in untreated leaves of young (26 +/- 28 ng g^{-1} frozen weight; Fig 5.2E) and mature plants (25 +/- 25 ng g^{-1} frozen weight). Overall, IAA levels did not change significantly in young or mature Col-0 in response to *Pst* compared to untreated plants. IAA-Asp is reported as a relative amount (peak area) because there was no stable isotopic internal standard available for doing absolute quantification. Similar levels of IAA-Asp were detected in untreated young plants (2290 +/- 257 peak area g^{-1} frozen weight; Fig 5.2F) and untreated mature plants (1060 +/- 1500 peak area g^{-1} frozen weight). At 48 hpi, IAA-Asp levels were similar to untreated samples in young and mature plants. At 72 hpi, IAA-Asp accumulation increased to similar levels in young (4030 +/- 1595 peak area g^{-1} frozen weight) and mature plants (3910 +/- 5527 peak area g^{-1} frozen weight). There was up to four-fold more IAA-Asp in young and mature plants after inoculation with *Pst* compared to untreated samples. ABA levels were similar in untreated young plants (4.2 +/- 6.0 ng g^{-1} frozen weight; Fig 5.2G) and untreated mature plants (7.1 +/- 10 ng g^{-1} frozen weight). There was no substantial increase in ABA levels at 48 or 72 hpi in young or mature plants. As a whole, these data suggest that IAA and ABA levels did not increase in young and mature plants after inoculation with *Pst*, and SA and IAA-Asp accumulated to similar levels in young plants compared to mature plants after inoculation with *Pst*.

The phytohormone analyses presented thus far paint a picture where JA, OPDA, JA-Ile, SA, and IAA-Asp levels appear to be elevated to similar levels in young and mature plants in response to *Pst*, and young and mature plants accumulate little IAA and

ABA in response to *Pst* compared to untreated samples. Therefore, the increase in expression of some defense genes observed during ARR (Chapter 3; this Chapter) does not seem to be associated with increased accumulation of these phytohormones at 48 or 72 hpi.

However, the elevated expression of *PR1* that was observed during early stages of ARR (before 24 hpi) in a preliminary experiment (Fig 5.1) may be the result of elevated accumulation of free SA before 24 hpi in mature plants compared to young plants. To test this idea, free SA levels were quantified in young and mature Col-0 at different time points after inoculation with *Pst* (un, 5 min, 6, 12, 24, 48, and 72 hpi: Fig 5.3). Samples were collected from two independent experiments where five week-old Col-0 supported high *Pst* levels (> 10^6 cfu ld^{-1}), and six week-old Col-0 supported low *Pst* levels (< 5.0 x 10^5 cfu ld^{-1}). The data is presented as the average of two experiments (n=2 +/- standard deviation). SA levels were similar in untreated young and mature Col-0. There were similar levels of SA in young and mature plants at 5 minutes, 6, 12, and 24 hpi (young, 774 +/- 617 ng g^{-1} frozen weight; mature, 1050 +/- 30 ng g^{-1} frozen weight at 24 hpi). SA levels remained elevated in young plants at 48 hpi (749 +/- 73 ng g^{-1} frozen weight) and 72 hpi (950 +/- 371 ng g^{-1} frozen weight), whereas in mature plants SA levels declined at 48 hpi (459 +/- 19 ng g^{-1} frozen weight) and 72 hpi (453 +/- 80 ng g^{-1} frozen weight). There was two-fold less SA in mature compared to young plants at 48 hpi ($P < 0.05$, Student's T-test), however, there was no significant different in SA levels in young and mature plants and 72 hpi ($P = 0.15$, Student's T-test). Overall, SA accumulated in a

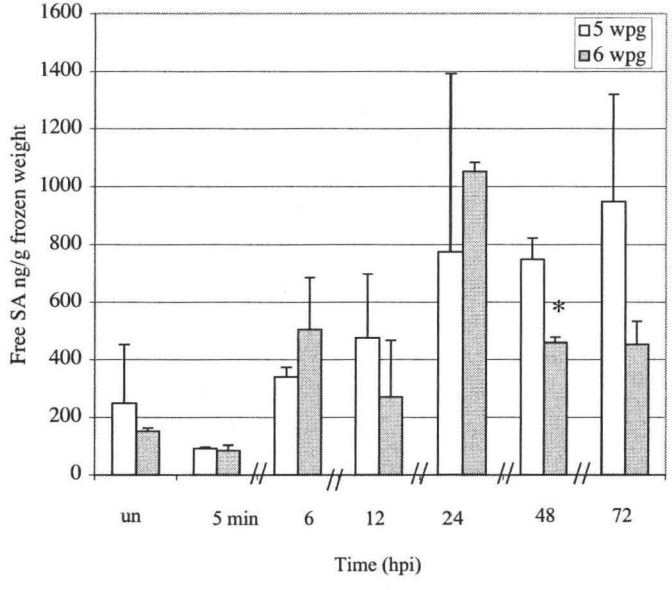


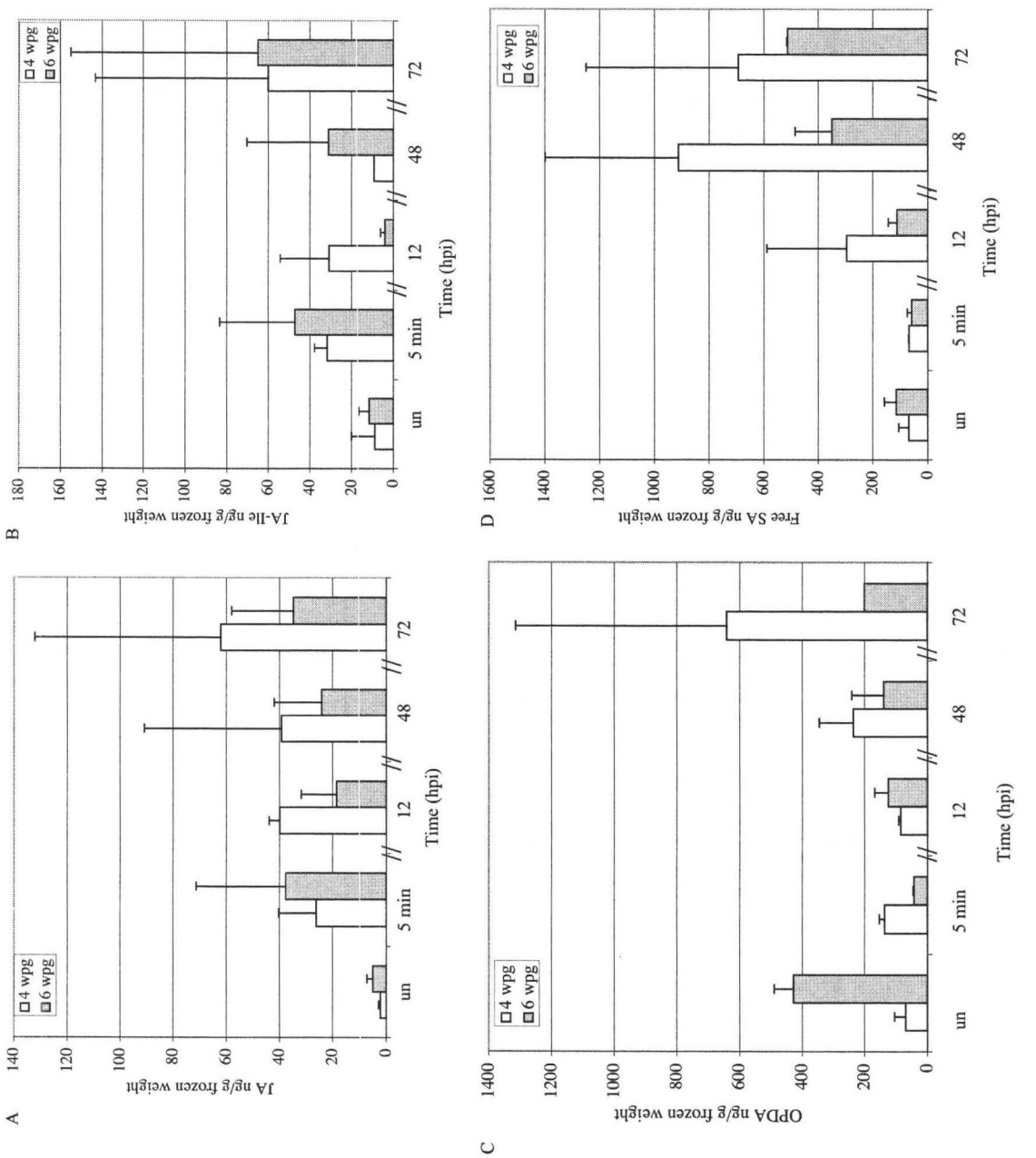
Figure 5.3. Free SA levels in leaves of ARR-incompetent Col-0 (5 wpg) and Col-0 plants exhibiting ARR (6 wpg). Untreated (un), 5 min, 6, 12, 24, 48, and 72 hpi (10^6 cfu ml^{-1} of *Pst*) samples were analyzed. Each data point is an average of one replicate from two independent experiments (n=2) +/- SD. * $P < 0.05$ Student's T-Test (see Appendix A Fig A44, Fig A45, Fig A46, Fig A47, Fig A50 and Fig A51 for replicates).

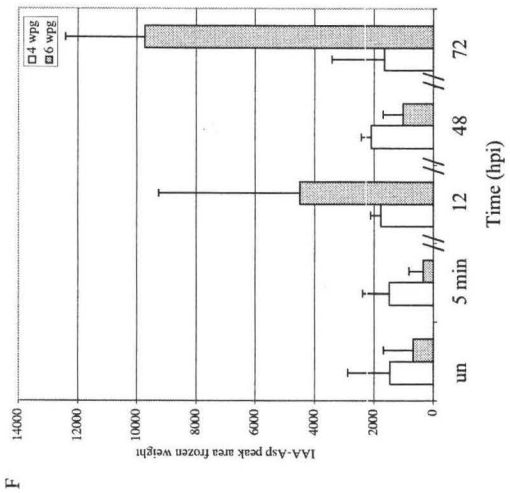
similar fashion in young and mature plants after inoculation with *Pst*.

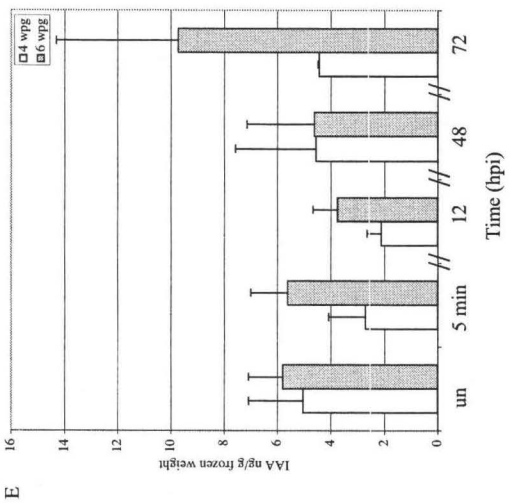
Expression of *PDF1.2a* during ARR (Chapter 3) does not seem to be associated with increased accumulation of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, or ABA at 48 or 72 hpi in mature compared to young plants. However, early expression of *PDF1.2a* (before 24 hpi) during ARR may be the result of higher levels of JA, JA-Ile, OPDA, and/or other phytohormones in mature compared to young plants before 24 hpi. This idea was tested by analyzing phytohormone levels (JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA) in samples collected from young and mature Col-0 at different time points after inoculation with *Pst* (un, 5 min, 12, 48, and 72 hpi: Fig 5.4). Samples were collected from two independent experiments where four week-old Col-0 supported high *Pst* levels ($> 10^6$ cfu ld^{-1}), and six week-old Col-0 supported low *Pst* levels ($< 5.0 \times 10^5$ cfu ld^{-1}). Similar levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA were detected in untreated young and mature plants and at 5 min and 12, 48, and 72 hpi. These levels were similar to what was observed previously (compare Fig 5.4 and Fig 5.2).

To determine if there is a difference in JA and SA levels in young and mature plants at 24 hpi, three biological replicates collected from ARR-incompetent young plants and mature plants exhibiting ARR were sent to the Plant Biotechnology Institute (PBI; Saskatoon, Saskatchewan) (Fig 5.5). PBI offers JA and SA analysis without analyzing other hormones. Samples were sent to PBI before we were aware of the hormone analysis services offered by the Donald Danforth Plant Science Center. JA (young, 1.0 +/- 0.4 μg

Figure 5.4. A) JA, B) JA-Ile, C) OPDA, D) free SA, E) IAA, F) IAA-Asp, and G) ABA levels in leaves of young (4 wpg) and mature (6 wpg) Col-0. Untreated (un), 5 min, 12, 48, and 72 hpi (10^6 cfu ml^{-1} of *Pst*) samples were analyzed. Each data point is the average of one replicate from two independent experiments (n=2) +/- SD (see Appendix A Fig A46, Fig A48, Fig A49, Fig A50, and Fig A51 for replicates).


A
JA ng/g frozen weight
0
20
40
60
80
100
120
140
4 wpg
6 wpg
un
5 min
12
48
72
Time (hpi)
B
JA-Ile ng/g frozen weight
0
20
40
60
80
100
120
140
160
180
4 wpg
6 wpg
un
5 min
12
48
72
Time (hpi)
C
OPDA ng/g frozen weight
0
200
400
600
800
1000
1200
1400
4 wpg
6 wpg
un
5 min
12
48
72
Time (hpi)
D
Free SA ng/g frozen weight
0
200
400
600
800
1000
1200
1400
1600
4 wpg
6 wpg
un
5 min
12
48
72
Time (hpi)


F
IAA-Asp peak area frozen weight
14000
12000
10000
8000
6000
4000
2000
0
4 wpg
6 wpg
un
5 min
12
48
72
Time (hpi)


E
IAA ng/g frozen weight
16
14
12
10
8
6
4
2
0
4 wpg
6 wpg
un
5 min
12
48
72
Time (hpi)

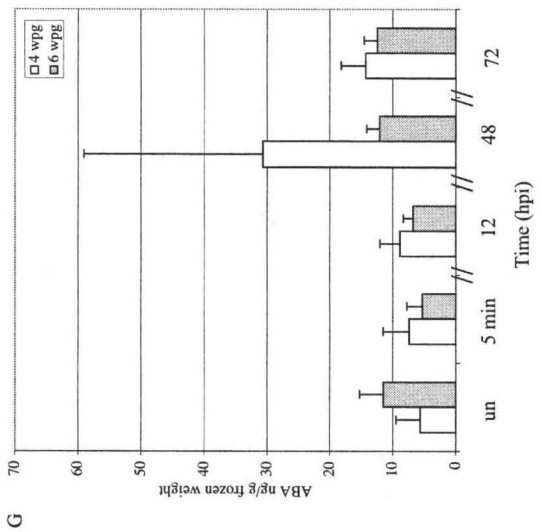
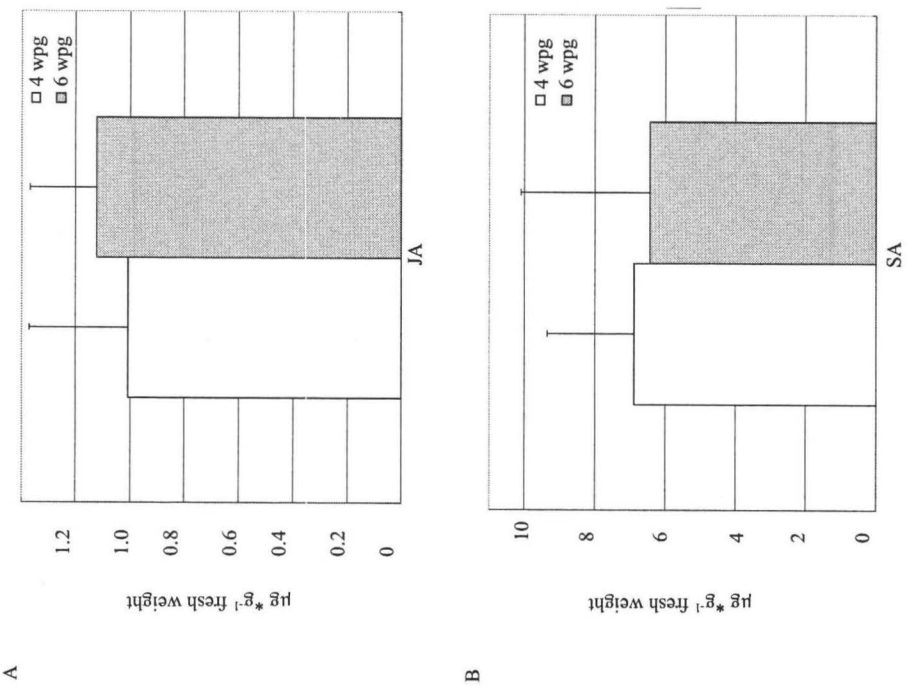

G
ABA ng/g frozen weight
70
60
50
40
30
20
10
0
4 wpg
6 wpg
un
5 min
12
48
72
Time (hpi)

Figure 5.5. : Free A) JA and B) SA levels in leaves collected from young (4 wpg) and mature (6 wpg) Col-0 after 24 hpi with *Pst* (10^6 cfu ml^{-1}). The average of three samples (n=3) +/- SD is shown. Each sample contains leaves from at least six plants.


A
μg *g-1 fresh weight
1.2
1.0
0.8
0.6
0.4
0.2
0
4 wpg
6 wpg
JA
B
μg *g-1 fresh weight
10
8
6
4
2
0
4 wpg
6 wpg
SA

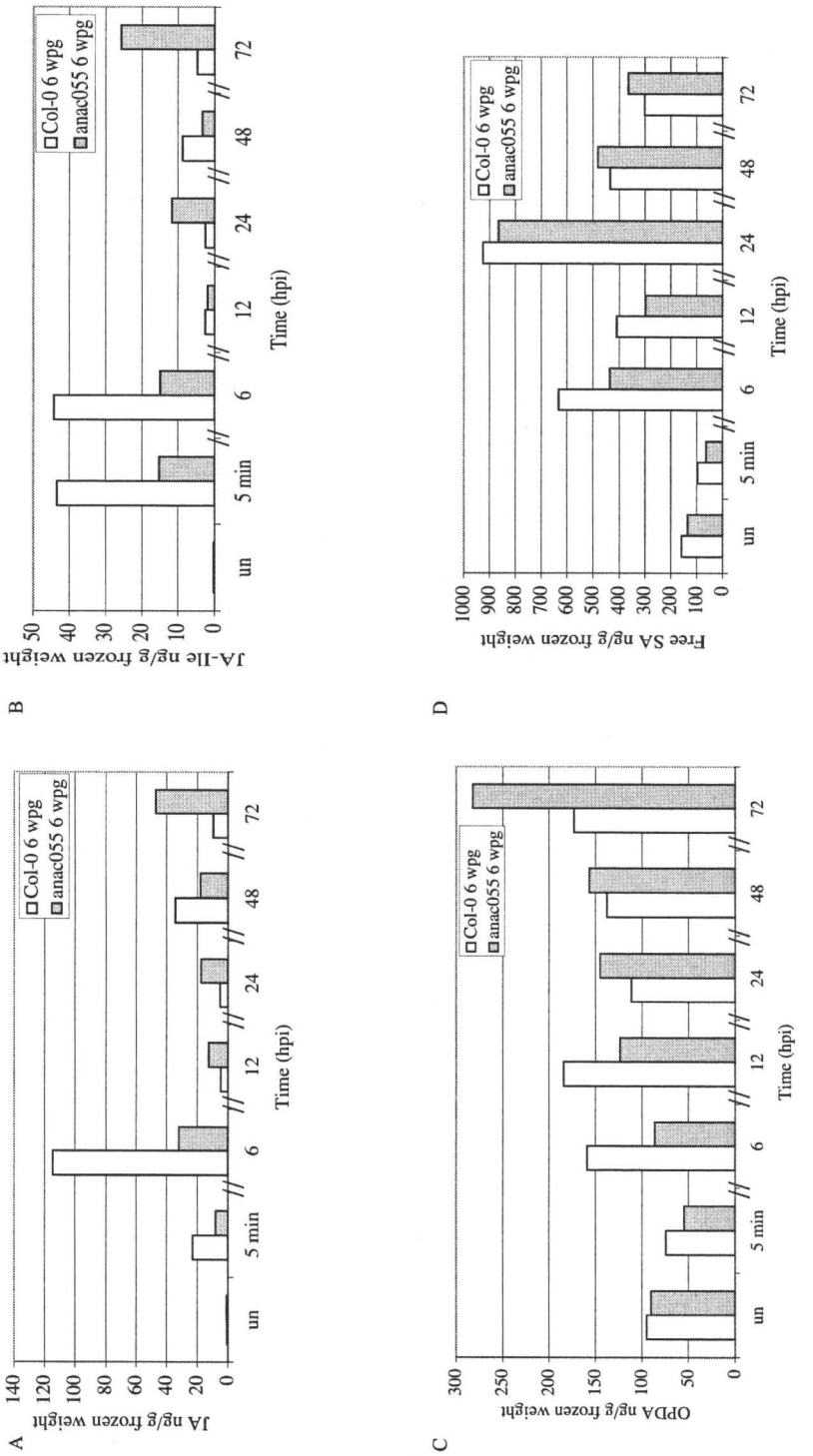
g^{-1} fresh weight; mature 1.1 +/- 0.2 μg g^{-1} fresh weight) and SA (young, 6.9 +/- 2.5 μg g^{-1} fresh weight; mature 6.4 +/- 3.7 μg g^{-1} fresh weight) accumulated to similar levels in young and mature plants at 24 hpi. Ten-fold more JA and six-fold more SA were observed in this analysis compared to analysis performed by the Donald Danforth Center (discussed later) (compare JA levels in Fig 5.2A and Fig 5.5A, and SA levels in Fig 5.3 and Fig 5.5B).

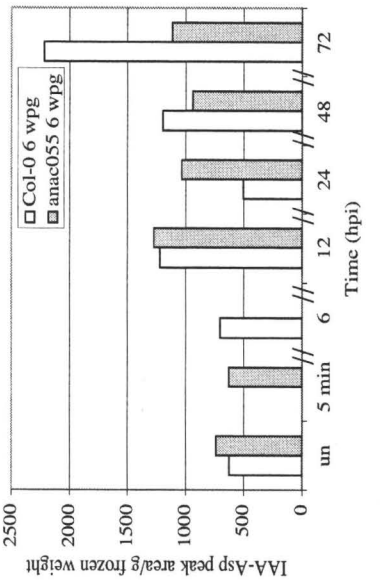
The phytohormone analyses presented here show that JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA accumulate to similar levels in young and mature plants after inoculation with *Pst*.

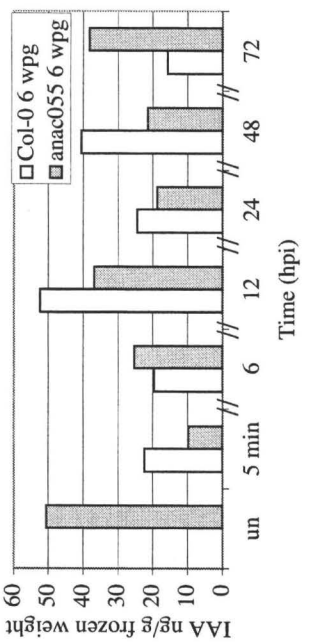
5.4c The ARR defect of *anac055* is associated with wild-type levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA

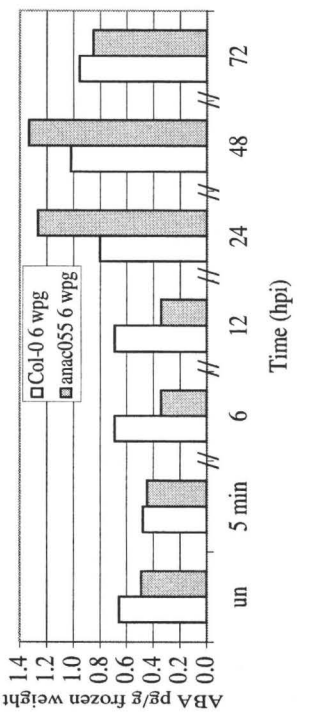
The ARR defect of *anac055* is associated with attenuated expression of *Lipoxygenase2* (*LOX2*: a JA biosynthesis gene) and *Isochorismate Synthase1* (*ICS1*: a SA biosynthesis gene) compared to Col-0 (Chapter 3), suggesting that ANAC055 contributes to regulation of JA and SA biosynthesis during ARR. Therefore, mature *anac055* may have lower levels of SA and JA after inoculation with *Pst* compared to Col-0. To test this idea and to see if mature *anac055* is compromised in accumulating other hormones after inoculation with *Pst*, JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA levels were measured in Col-0 and *anac055*. Samples were collected from mature, six week-old Col-0 and *anac055* at different time points after inoculation with *Pst* (untreated, 5 min, 6, 12, 24, 48, and 72 hpi: Fig 5.6). Only one replicate per time point was available for this preliminary analysis. In this experiment, mature Col-0 supported low *Pst* levels

Figure 5.6. Preliminary results showing (A) JA, (B) JA-Ile, (C) OPDA, (D) free SA, (E) IAA, (F) IAA-Asp, and (G) ABA levels in leaves of mature (6 wpg) Col-0 and *anac055*. Untreated (un), 5 min, 6, 12, 24, 48, and 72 hpi (10^6 cfu ml^{-1} of *Pst*) samples were analyzed. No error bars are included since only one replicate per genotype was analyzed for each time point.


A
JA ng/g frozen weight
140
120
100
80
60
40
20
0
Col-0 6 wpg
anac055 6 wpg
un
5 min
6
12
24
48
72
Time (hpi)
B
JA-Ile ng/g frozen weight
50
40
30
20
10
0
Col-0 6 wpg
anac055 6 wpg
un
5 min
6
12
24
48
72
Time (hpi)
C
OPDA ng/g frozen weight
300
250
200
150
100
50
0
Col-0 6 wpg
anac055 6 wpg
un
5 min
6
12
24
48
72
Time (hpi)
D
Free SA ng/g frozen weight
1000
900
800
700
600
500
400
300
200
100
0
Col-0 6 wpg
anac055 6 wpg
un
5 min
6
12
24
48
72
Time (hpi)


F
IAA-Asp peak area/g frozen weight
2500
2000
1500
1000
500
0
un
5 min
6
12
24
48
72
Time (hpi)
Col-0 6 wpg
anac055 6 wpg


E
IAA ng/g frozen weight
60
50
40
30
20
10
0
un
5 min
6
12
24
48
72
Time (hpi)
Col-0 6 wpg
anac055 6 wpg


G
ABA pg/g frozen weight
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
un
5 min
6
12
24
48
72
Time (hpi)
Col-0 6 wpg
anac055 6 wpg

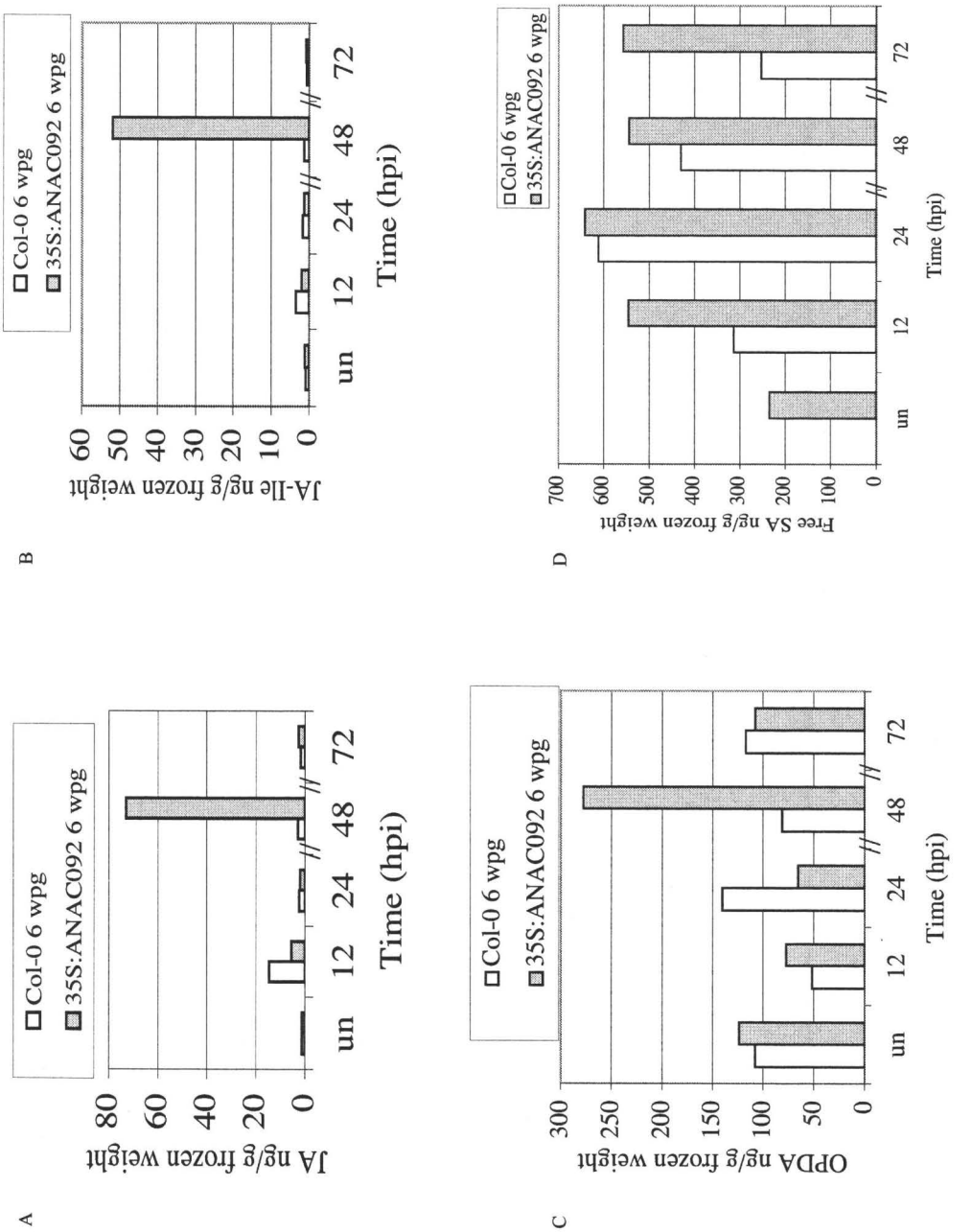
typical of plant exhibiting ARR (5.0 x 10^5 +/- 3.1 x 10^5 cfu ld^{-1}), whereas *anac055* supported three-fold higher *Pst* levels (1.4 x 10^6 +/- 9.0 x 10^5 cfu ld^{-1}; $P < 0.07$, Student's T-test). During ARR in Col-0, JA levels were elevated at 6 hpi (115 ng g^{-1} frozen weight), and JA-Ile levels were elevated at 5 min and 6 hpi (~ 44 ng g^{-1} frozen weight; Fig 5.6A,B). These spikes in JA and JA-Ile accumulation were transient and were not maintained after 12 hpi. JA and JA-Ile are known to accumulate in response to wounding (Farmer et al., 2003). Therefore, these spikes in JA and JA-Ile levels may be the result of a wounding response triggered in the leaves during the inoculation procedure (see 4.7 Materials and Methods). Overall, JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA levels were similar in mature *anac055* and wild-type plants after inoculation with *Pst*. This preliminary data suggests that, contrary to what was expected, the ARR defect in *anac055* is not associated with reduced accumulation of JA, JA-Ile, OPDA, or SA.

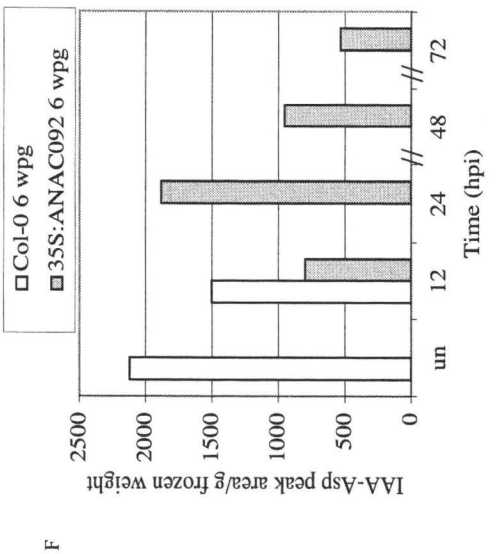
5.4d The ARR defect of 35S:*ANAC092* is associated with wild-type levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA

Similar to *anac055*, 35S:*ANAC092* exhibits an ARR defect that is associated with reduced *LOX2* expression compared to the wild-type (Chapter 3). This suggests that ANAC092 is a negative regulator of JA biosynthesis during ARR. Therefore, 35S:*ANAC092* may accumulate lower JA levels compared to Col-0 during ARR. To test this prediction, and to determine if the ARR defect of 35S:*ANAC092* is associated with compromised accumulation of other phytohormones, JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA levels were quantified during ARR in mature, six week-old Col-0 and 35S:*ANAC092* (untreated, 12, 24, 48, and 72 hpi). Only one replicate per time point was

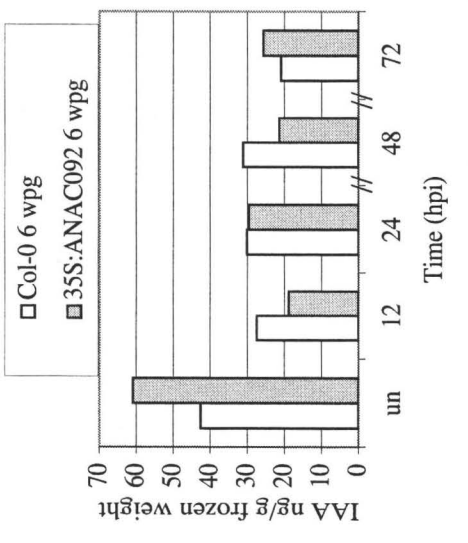
available for this preliminary analysis. Mature Col-0 supported low levels of *Pst* typical of plants exhibiting ARR (7.9×10^4 +/- 1.3×10^4 cfu ld^{-1}), whereas 35S:*ANAC092* supported three-fold higher *Pst* levels compared to Col-0 (2.1×10^5 +/- 6.6×10^4 cfu ld^{-1}; $P < 0.05$, Student's T-test). Col-0 and 35S:*ANAC092* accumulated low levels of JA, JA-Ile, OPDA, IAA, and ABA during ARR (similar to levels observed in untreated leaves; Fig 5.7A, B, C, E, G). There was a spike in JA, JA-Ile, OPDA, and ABA levels at 48 hpi in 35S:*ANAC092* but this increase was not maintained at 72 hpi. As seen before (Fig 5.3), SA levels were elevated at 24 hpi (610 ng g^{-1} frozen weight), and they subsequently declined at 48 and 72 hpi during ARR in Col-0 (253 ng g^{-1} frozen weight at 72 hpi: Fig 5.7D). 35S:*ANAC092* had similar levels of SA as the wild-type after inoculation with *Pst* (Fig 5.7D). In Col-0, the level of IAA-Asp fell below detectable levels at 24, 48, and 72 hpi, whereas 35S:*ANAC092* had elevated levels of IAA-Asp at 12 hpi (799 peak are g^{-1} frozen weight) and 24 hpi (1880 peak are g^{-1} frozen weight). However, IAA-Asp levels subsequently decreased in 35S:*ANAC092* at 48 hpi (953 peak are g^{-1} frozen weight) and 72 hpi (534 peak area g^{-1} frozen weight), but they remained higher than levels observed in Col-0. IAA-Asp accumulated to lower levels in Col-0 in this ARR experiment than what was previously observed (compare Fig 5.7F and Fig 5.2F). Taken together, this preliminary data suggests that the ARR defect of 35S:*ANAC092* is associated with wild-type levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp and ABA.

Figure 5.7. Preliminary results showing (A) JA, (B) JA-Ile, (C) OPDA, (D) SA, (E) IAA, (F) IAA-Asp, and (G) ABA levels in leaves of mature (6 wpg) Col-0 and 35S:*ANAC092*. Untreated (un), 12, 24, 48, and 72 hpi (10^6 cfu ml^{-1} of *Pst*) samples were analyzed. No error bars are included since only one replicate per genotype was analyzed for each time point.


A
JA ng/g frozen weight
0
20
40
60
80
un
12
24
48
72
Time (hpi)
Col-0 6 wpg
35S:ANAC092 6 wpg
B
JA-Ile ng/g frozen weight
0
10
20
30
40
50
60
un
12
24
48
72
Time (hpi)
Col-0 6 wpg
35S:ANAC092 6 wpg
C
OPDA ng/g frozen weight
0
50
100
150
200
250
300
un
12
24
48
72
Time (hpi)
Col-0 6 wpg
35S:ANAC092 6 wpg
D
Free SA ng/g frozen weight
0
100
200
300
400
500
600
700
un
12
24
48
72
Time (hpi)
Col-0 6 wpg
35S:ANAC092 6 wpg


Col-0 6 wpg
35S:ANAC092 6 wpg
IAA-Asp peak area/g frozen weight
2500
2000
1500
1000
500
0
un
12
24
48
72
Time (hpi)

F


Col-0 6 wpg
35S:ANAC092 6 wpg
IAA ng/g frozen weight
70
60
50
40
30
20
10
0
un
12
24
48
72
Time (hpi)

E

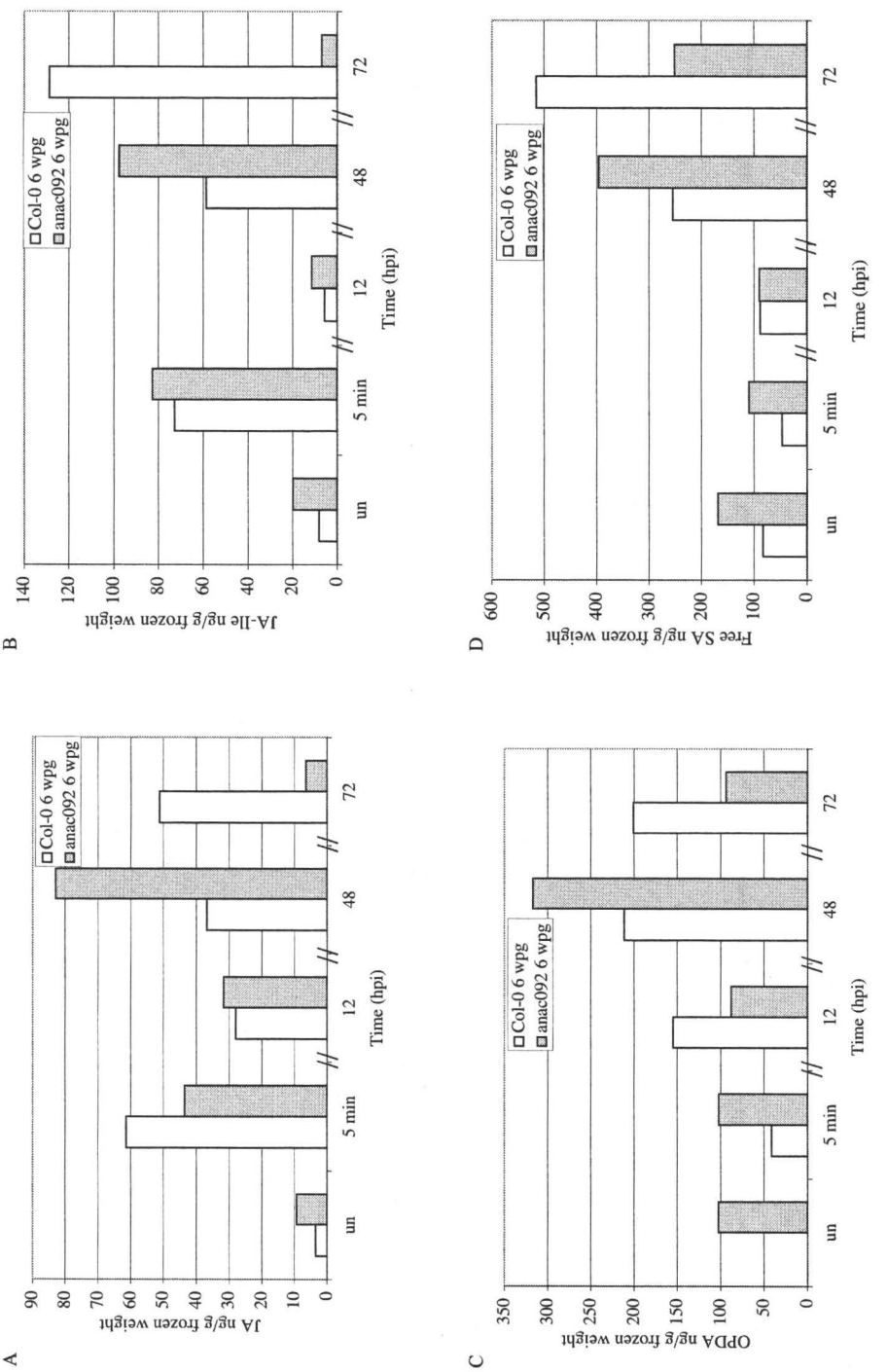
Col-0 6 wpg
35S:ANAC092 6 wpg
ABA pg/g frozen weight
5.0
4.0
3.0
2.0
1.0
0.0
un
12
24
48
72
Time (hpi)

G

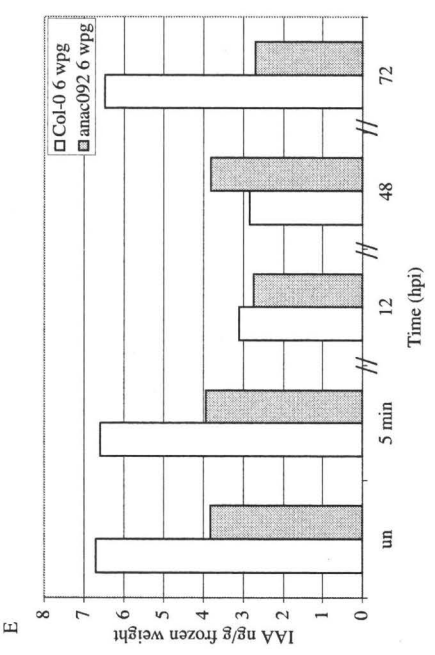
5.4e The ARR defect of *anac092* is associated with wild-type levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA

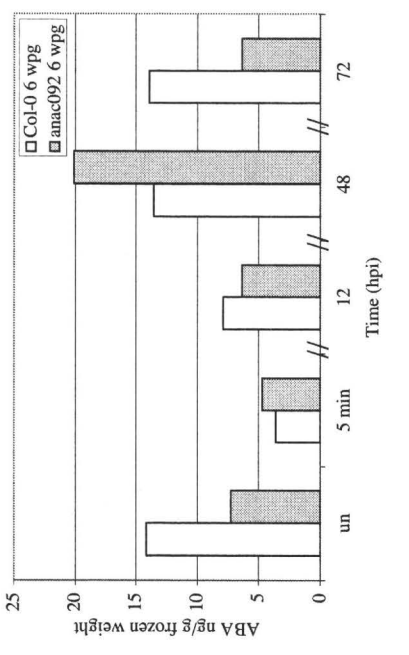
anac092 displays an ARR defect that is associated with delayed flowering, suggesting that the onset of ARR is delayed in *anac092* (Chapter 3). To determine if the ARR defect of *anac092* is associated with altered levels of phytohormones, JA, JA-Ile, OPDA SA, IAA, IAA-Asp, and ABA levels were measured in mature *anac092* and wild-type plants after inoculation with *Pst*. Leaves of mature, six week-old Col-0 and *anac092* were collected from an ARR experiment where Col-0 plants exhibiting ARR supported low levels of *Pst* (9.6×10^4 +/- 5.1×10^4 cfu ld^{-1}) and six week-old *anac092* supported six-fold higher *Pst* levels (5.2×10^5 +/- 3.1×10^5 cfu ld^{-1}; $P < 0.05$ Student's T-test) (untreated, 12, 48, and 72 hpi) (Fig 5.8). Mature *anac092* and wild-type plants had similar levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA after inoculation with *Pst*. This preliminary data suggests that the ARR defect of *anac092* is associated with wild-type levels of these phytohormones.

Figure 5.8. Preliminary results showing (A) JA, (B) JA-Ile, (C) OPDA, (D) SA, (E) IAA, (F) IAA-Asp, and (G) ABA levels in leaves of mature (6 wpg) Col-0 and *anac092*. Untreated (un), 5 min, 12, 24, 48, and 72 hpi (10^6 cfu ml^{-1} of *Pst*) samples were analyzed. No error bars are included since only one replicate per genotype was analyzed for each time point.


A
JA ng/g frozen weight
Col-0 6 wpg
anac092 6 wpg
un
5 min
12
48
72
Time (hpi)
B
JA-Ile ng/g frozen weight
Col-0 6 wpg
anac092 6 wpg
un
5 min
12
48
72
Time (hpi)
C
OPDA ng/g frozen weight
Col-0 6 wpg
anac092 6 wpg
un
5 min
12
48
72
Time (hpi)
D
Free SA ng/g frozen weight
Col-0 6 wpg
anac092 6 wpg
un
5 min
12
48
72
Time (hpi)


F
IAA-Asp peak area/g frozen weight
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Col-0 6 wpg
anac092 6 wpg
un
5 min
12
48
72
Time (hpi)


E
IAA ng/g frozen weight
8
7
6
5
4
3
2
1
0
Col-0 6 wpg
anac092 6 wpg
un
5 min
12
48
72
Time (hpi)


G
ABA ng/g frozen weight
25
20
15
10
5
0
Col-0 6 wpg
anac092 6 wpg
un
5 min
12
48
72
Time (hpi)

5.5 Discussion

5.5a Young and mature plants have similar levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA after inoculation with *Pst*

Analysis of *in planta* levels of a number of hormones, including JA, JA-Ile, OPDA, IAA, IAA-Asp, and ABA, was undertaken to investigate the difference in hormone profiles of young and mature plants after inoculation with *Pst*. This data should be considered with caution as the large standard deviation values demonstrate that there is great variation in phytohormone levels between experiments. Young and mature plants accumulate similar levels of JA, JA-Ile, OPDA, SA, and IAA-Asp after inoculation with *Pst*. Additionally, IAA and ABA levels do not rise in response to *Pst* in either young or mature plants. Collectively, this data suggests that levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA are not elevated in mature compared to young plants after inoculation with *Pst*. Therefore, higher expression levels of some JA/ET-associated genes during ARR (Chapter 2; Chapter 3; Carviel et al., 2009) do not appear to be associated with greater accumulation of these phytohormones in mature plants compared to young plants after inoculation with *Pst*.

It is difficult to compare the absolute phytohormone levels presented in this chapter with data from the literature as most labs use different inoculum concentrations (10^6 cfu ml^{-1} to 10^8 cfu ml^{-1}), different inoculation techniques (foliar spray versus pressure infiltration used here), different strains of virulent and avirulent *Pseudomonas*, and different Arabidopsis ecotypes. Also, different laboratories grow their plants under different conditions (length of photoperiod), and use different phytohormone

quantification methods. However, reports that quantified phytohormone levels in Arabidopsis after inoculation with virulent *Pseudomonas* observe that there is an increase in JA (Schmelz et al., 2003; Spoel et al., 2003; Nickstadt et al., 2004; Block et al., 2005; de Torres-Zabala et al., 2007; de Torres-Zabala et al., 2009), ABA (Schmelz et al., 2003; de Torres-Zabala et al., 2007; de Torres-Zabala et al., 2009), and OPDA (Block et al., 2005) levels in young plants. The absolute levels of phytohormones, however, vary considerably between studies. For example, Schmelz et al. (2003) reported 1000 ng g^{-1} fresh weight of JA and 70 ng g^{-1} fresh weight of ABA accumulation at 48 hpi, while de Torres Zabala et al. (2009) reported 4000 ng g^{-1} fresh weight of JA and 1200 ng g^{-1} fresh weight of ABA accumulation at 22 hpi. de Torres-Zabala et al. (2007) reported 500 ng g^{-1} fresh weight of JA and 40 ng g^{-1} fresh weight of ABA accumulation at 18 hpi, and Block et al. (2005) reported 300 ng g^{-1} fresh weight of JA at 72 hpi with *Pst*. Our analysis shows that 100 ng g^{-1} frozen weight (Donald Danforth Center) to 1000 ng g^{-1} fresh weight (PBI) of JA and 10 ng g^{-1} frozen weight of ABA accumulated in young plants after inoculation with *Pst*.

The JA and ABA levels obtained from the Donald Danforth Center are considerably less than what was previously reported. However, Schmelz et al. (2003), de Torres-Zabala et al (2007), and de Torres-Zabala et al. (2009) inoculated their plants with 10^8 cfu ml^{-1} *Pst*, and Block et al. (2005) inoculated their plants with 10^7 cfu ml^{-1} *Pst*, whereas we used 10^6 cfu ml^{-1} *Pst*. Higher levels of ABA accumulate in young plants when they are inoculated with higher concentrations of *Pst* (de Torres-Zabala et al., 2007). Therefore, the higher levels of JA and ABA reported by the studies mentioned

above may be a result of the higher inoculum concentration used in those experiments. Also, whereas the studies discussed here present their data as the average of biological replicates from the same experiment, the phytohormone data included in this chapter represent the average of a number of independent experiments. In addition, ion suppression was seen in the analysis done by the Donald Danforth Center (see Material and Methods section 5.6d Phytohormone Analysis). Ion suppression occurs in the ionization step of mass spectrometry when one eluted component influences the ionization of a co-eluted compound (Buhrman et al., 1996). This can result in under- or over-reporting of co-eluted chemicals. Therefore, the low JA and ABA levels detected by the Donald Danforth Center may have been a result of ion suppression. Unlike JA and ABA, we report higher levels of OPDA (900 ng g^{-1} frozen weight) compared to what was previously reported by Block et al. (2005) (400 ng g^{-1} frozen weight) at 72 hpi. This may also have been a result of ion suppression. Overall, our data is consistent with reports in the literature that show an increase in JA and OPDA levels in response to *Pst* in young plants, however we do not observe an increase in ABA levels above those observed in untreated samples.

IAA/auxin is involved in negatively regulating resistance to *Pst* in Arabidopsis by promoting the down-regulation of SA defense responses (Bari and Jones, 2009; Grant and Jones, 2009). The mutually antagonistic relationship between IAA and SA is exploited by some pathogens to facilitate colonization of plants. The AvrRpt2 effector of avirulent *Pseudomonas* strains, for example, up-regulates auxin levels in Arabidopsis, and this is thought to down-regulate SA defense responses (Chen et al., 2007). Our

analysis shows that IAA levels do not increase in young or mature plants in response to virulent *Pst*. This is in contradiction to the increase in IAA levels (10 to 35 ng g^{-1} fresh weight) in young Arabidopsis responding to virulent *Pst* at 48 hpi that was reported in other studies (Schmelz et al. 2003; Chen et al., 2007). However, both of these studies used a *Pst* inoculum concentration of 10^8 cfu ml^{-1} whereas we used 10^6 cfu ml^{-1}. Young plants may accumulate more IAA as a result of an increase in the concentration of *Pst* inoculum, in a similar fashion to ABA (de Torres-Zabala et al., 2007). This difference in methodologies may account for the discrepancies in IAA levels between our data and their data. Alternatively, ion suppression may also be responsible for the low levels of IAA detected in our analyses. We also show that IAA-Asp accumulates to similar levels in young and mature plants in response to *Pst*. This IAA conjugate is thought to be an intermediate in a pathway that leads to IAA catabolism and degradation (Ljung et al., 2002). Collectively, this data suggests that IAA production and degradation are not altered during ARR in mature plants compared to young plants after inoculation with *Pst*.

5.5b The role of SA in ARR

PR1 was previously shown to be expressed to lower levels in mature compared to young plants during late stages of infection with *Pst* (after 24 hpi; Kus et al., 2002; Rusterucci et al., 2005). To determine if *PR1* is up-regulated before 24 hpi during ARR, *PR1* expression was monitored in young and mature plants at 5 min, 12, 24, and 48 hpi. Preliminary results suggest that *PR1* expression is up-regulated to a greater extent in mature compared to young plants during early stages of infection with *Pst* (before 24 hpi). At 24 and 48 hpi, however, *PR1* is expressed to lower levels in mature compared to

young plants. The down-regulation of *PR1* expression during late stages of infection with *Pst* (after 24 hpi) observed here is similar to previously published data (Kus et al., 2002; Rusterucci et al., 2005). Kus et al. (2002) also observed that Non-expresser of PR genes1 (NPR1), a central regulator of expression of *PR1* (Dong, 2004), is not required for ARR. This suggests that the *PR1* expression observed during ARR is independent of NPR1. As *PR1* is a marker for SA signaling, increased expression of *PR1* during early stages of ARR could be due to elevated levels of SA in that time period in mature compared to young plants. Therefore, free SA levels were quantified in young and mature plants after inoculation with *Pst*. SA levels increase in a similar manner in young and mature plants before 24 hpi, and they reach a similar level at 24 hpi. This suggests that elevated *PR1* expression during early stages of ARR is not associated with elevated levels of SA in mature compared to young plants after inoculation with *Pst*. Expression of *PR1* during early stages of ARR (preliminary data) may be a result of hypersensitivity to SA. In other words, although mature plants do not seem to accumulate more SA than young plants after inoculation with *Pst*, they may be more sensitive to SA and initiate some SA signaling earlier than young plants. Alternatively, another phytohormone may contribute to up-regulation of expression of *PR1* during early stages of ARR.

The level of SA accumulation in young plants reported here (~800 ng g^{-1} frozen weight at 24 hpi reported by Donald Danforth Center and ~7000 ng g^{-1} fresh weight at 24 hpi reported by PBI) is comparable to some studies where SA accumulation ranged between 700 ng g^{-1} fresh weight at 18 hpi (de Torres-Zabala et al., 2007) and 1000 ng g^{-1} fresh weight at 24 and 48 hpi in young plants (Cameron et al., 1999; Schmelz et al.,

2003), but it is lower than other studies that reported 20 000 ng g^{-1} fresh weight of SA accumulation at 22 hpi in young plants (de Torres Zabala et al., 2009). As discussed in the previous section, this could be due to the differences in *Pst* inoculum concentrations used in this study (10^6 cfu ml^{-1}) compared to the other studies (10^8 cfu ml^{-1}), or it may be due to ion suppression.

The ARR defect of *anac055* is associated with attenuated expression of *ICS1* (Chapter 3), but it is not associated with less SA accumulation than the wild-type according to preliminary data. SA accumulation in *anac055* may be the result of ICS1 enzyme activity that is independent of *ICS1* expression levels. Alternatively, the second SA biosynthetic pathway, the Phenylalanine Ammonia Lyase (PAL) pathway (Wildermuth, 2006), may contribute to this SA accumulation.

The SA analyses presented in this chapter included only free SA that was quantified in the entire leaf without making any distinction between inter- and intracellular SA. As intercellular SA plays an important role during ARR (Cameron and Zaton, 2004; Carviel et al., 2009), the ARR defects of *anac055* and 35S:*ANAC092* may be associated with less SA in the intercellular space than Col-0. Such a difference in intercellular SA may not be apparent when SA levels are quantified in the entire leaf as was done here. Also, SA derivatives were not measured in these assays. Some SA derivatives, such as SA-glucoside (SAG), play important roles in SA storage (Vlot et al., 2009). SAG production may influence the level of free SA in plants. For example, production of SAG, by conjugating a glucose molecule to free SA, during ARR in mature plants might result in lower amounts of free SA. Other SA derivatives, such as gentisic

acid (2,5-dihydroxybenzoic acid), contribute to some defense signaling (Vlot et al., 2009). For example, treating tomato plants with gentisic acid was found to induce accumulation of some PR proteins (Belles et al., 1999). Increased accumulation of some SA derivatives in plants exhibiting ARR compared to young plants after inoculation with *Pst* may contribute to expression of *PR1* during ARR.

5.5c The role of JA in ARR

Mature plants express some JA/ET-related genes to higher levels than young plants after inoculation with *Pst* (Chapter 2; Chapter 3; Carviel et al., 2009). However, young and mature plants have similar levels of JA, JA-Ile, and OPDA after inoculation with *Pst*. Previous data illustrate that the JA biosynthesis-deficient mutant *lox2*, and the JA signaling mutants *jin1* and *jar1* exhibit wild-type ARR (Chapter 3; Carviel et al., 2009). Also, a number of components of the JA pathway, including COI1 and OPR3, may be involved in negatively regulating ARR, as preliminary data illustrates that *coi1* and *opr3* plants exhibit an enhanced ARR response (Chapter 4). Furthermore, gene expression analysis shows that expression of *ANAC055* and *ANAC092* is modestly elevated in mature *lox2* and preliminary data illustrates that *ANAC092* expression is up-regulated in mature *coi1* after inoculation with *Pst* compared to the wild-type, suggesting that JA signaling is involved in negatively regulating *NAC* gene expression during ARR (Chapter 4). Collectively, these observations suggest that some components of JA signaling negatively regulate some components of the ARR pathway. However, ARR requires some JA signaling-associated genes, including *ANAC055* (Chapter 2) and

Ethylene Insensitive2 (Chapter 3). This suggests that JA signaling may be a positive and negative regulator of ARR.

Up-regulation of some JA/ET-associated genes during ARR (Chapter 2; Chapter 3) is not associated with elevated JA, JA-Ile, or OPDA levels compared to young plants. Up-regulation of some JA/ET-associated genes in mature compared to young plants after inoculation with *Pst* may be a result of hypersensitivity to JA and/or JA-related hormones. In other words, although mature plants do not seem to accumulate more JA, JA-Ile, or OPDA than young plants after inoculation with *Pst*, they may be more sensitive to these phytohormones. Hypersensitivity to JA, JA-Ile, and/or OPDA in mature plants may cause higher expression of some JA/ET-associated genes in mature plants than in young plants. Alternatively, up-regulation of some JA/ET-associated gene expression may be associated with increased production of (an)other phytohormone(s). *ANAC055* and *ANAC092* expression during ARR seems to be dependent on EIN2 (Chapter 3). EIN2 regulates JA- and ET-dependent pathways (Alonso et al., 1999). Therefore, increased expression of some JA/ET-related genes during ARR may be associated with elevated levels of ET. Moreover, other derivatives of JA that were not quantified in our assays, including methyl-JA (MeJA), may accumulate to greater levels in mature compared to young plants after inoculation with *Pst* and contribute to up-regulating some JA/ET signaling during ARR. Also, *Pst* may produce more coronatine, a JA mimic, in mature than in young plants, and this might contribute to increased expression of some JA/ET-associated genes during ARR.

5.5d The role of ANAC055 and ANAC092 in ARR

Gene expression data from previous chapters suggest that ANAC055 is a positive regulator of *LOX2*, *PDF1.2a*, and *ICS1* expression and ANAC092 is a negative regulator of *LOX2* and *PDF1.2a* expression during ARR (Chapter 3). In this chapter, preliminary data revealed that mature *anac055* and 35S:*ANAC092* have wild-type levels of JA, JA-Ile, and OPDA. This suggests that ANAC055 and ANAC092 do not regulate JA accumulation during ARR. ANAC055 also does not seem to be a regulator of SA accumulation during ARR, as the ARR defect of *anac055* is associated with wild-type levels of SA (preliminary data).

anac092 exhibits an ARR defect at six wpg that is associated with delayed flowering, suggesting that the onset of ARR is delayed in this mutant (Chapter 3). The preliminary data presented in this chapter shows that the ARR defect exhibited by *anac092* is associated with wild-type levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA. This suggests that the delay in the onset of ARR that seems to occur in *anac092* is not associated with altered levels of these phytohormones, and that ANAC092 regulates the onset of ARR by activating signaling that is downstream or independent of these phytohormones.

5.6 Materials and Methods

5.6a Plant Growth Conditions

The *Arabidopsis thaliana* wild-type Columbia (Col-0) was used along with *anac055* (SALK_014331), *anac029* (SALK_005010C), and 35S:*ANAC092* plants (line 1, S-Y. Chen, Chinese Academy of Sciences, China). Seeds were surface sterilized and

germinated on Murashige and Skoog medium, grown under continuous light (100 $\mu m^{-2}s^{-1}$) for 7 days, then the seedlings were transferred to soil (Sunshine Mix No. 1, Sun Gro Horticulture, Bellevue, WA) moistened with 1 g L^{-1} 20-20-20 fertilizer and grown at 21 to 23 °C with a light intensity of 150 to 200 $\mu m^{-2}s^{-1}$ maintaining the humidity at 85% under short days (9 hrs light and 15 hrs of darkness).

5.6b *In planta Pst* Growth Assays

Arabidopsis plants were inoculated with virulent *Pseudomonas syringae* pv. *tomato* (*Pst)* strain DC3000 (rifampicin and kanamycin resistant) obtained from Dr. Andrew Bent (University of Wisconsin at Madison) (Whalen et al., 1991). *Pst* was grown to mid-log phase in King's B media and kanamycin (50 $\mu g\ ml^{-1}$) (Sigma), shaken over-night at room temperature (22 to 25 °C), then diluted to 10^6 colony forming units (cfu ml^{-1}) in 10 mM $MgCl_2$. The *Pst* culture was pressure infiltrated into the abaxial sides of 8 to 10 leaves per plant using a needless 10 ml syringe, filling the intercellular space of the entire leaf. Leaves on plants without visible inflorescence stems were inoculated, and *in planta* bacterial growth was determined as previously described (Kus et al., 2002) with serial dilutions plated on King's B agar plates containing rifampicin (100 $\mu g\ ml^{-1}$) (Sigma) and kanamycin (50 $\mu g\ ml^{-1}$) (Sigma).

5.6c RT-PCR

Leaf samples were collected from Col-0 plants inoculated with *Pst* to analyze gene expression. Samples were collected from untreated plants and at 5 min, 12, 24, and 48 hpi from young (4 wpg) and mature (6 wpg) plants. RNA was extracted from leaf samples using the TRIzol method (Invitrogen), DNase treatment was performed with the

Ambion DNase Free system (Applied Biosystems), and cDNA was synthesized using the SuperScript III (Invitrogen) reverse transcriptase kit, according to the manufacturer's instructions. Standard PCR conditions were used with an annealing temperature of 60 °C for all primers (*PR1* At2g14610: F 5' AGACGCCAGACAAGTCACCGCTAC 3',
R 5' TCCCTCGAAAGCTCAAGATAGCCC 3';
ACTIN1 At2g37620: F 5' GGCGATGAAGCTCAATCCAAACG 3',
R 5' GGTCACGACCAGCAAGATCAAGACG 3') (The Institute for Molecular Biology and Biotechnology – MOBIX). *ACTIN1* was used as the constitutive internal control. All gels were stained with ethidium bromide.

5.6d Phytohormone Analysis

Samples were collected from untreated young (4 or 5 wpg) and mature (6 wpg) plants and at 5 min, 6, 12, 24, 48, and 72 hpi and flash frozen in liquid nitrogen. At least two leaves from different plants were included in each replicate. All samples were sent to The Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center (St. Louise, Missouri, USA) for simultaneous quantification of free JA, JA-Ile, OPDA, free SA, IAA, IAA-Asp, and ABA levels using liquid chromatography/mass spectroscopy/mass spectroscopy (LC/MS/MS).

LC-MS/MS analysis of acidic plant hormones

The method used for detection and quantification of acidic plant hormones was developed and performed by the Proteomics & Mass Spectrometry Facility at the Donald Danforth Plant Science Center. The method is similar to what is described by Chen et al. (2009), but modified to include additional plant hormone species. Briefly, samples were

ground in liquid nitrogen and internal standards (10 mL of 2.5 mM) were added. Samples were extracted with 1.5 mL acetonitrile/methanol (1:1 v:v). After lyophilization, samples were resolubilized in 200 mL of 50% MeOH. For LC separation, two monolithic C18 columns (Onyx, 4.6 mm x 100 mm, Phenomenex, CA, USA) with a guard cartridge was used flowing at 1 mL min^{-1}. The gradient was from 40% solvent A (0.1% (v:v) acetic acid in MilliQ water), held for 2 min, to 100% solvent B (90% acetonitrile (v:v) with 0.1% acetic acid (v:v) in 5 min. The LC was held at 100% B for 3 min and then ramped back to initial conditions and re-equilibrated for an additional 2 min. To minimize variation from the autosampler, the sample loop was overfilled with 52 mL of sample and the sample storage temperature was set to 8 °C. The LC-MS/MS system used is composed of a Shimadzu LC system with LEAP CTC PAL autosampler coupled to an Applied Biosystems 4000 QTRAP mass spectrometer equipped with a TurboIon Spray (TIS) electrospray ion source. Source parameters were set to: CUR: 25, GAS1: 50, GS2: 50 (arbitrary unit), CAD: high, IHE: on, TEM: 550 °C, IS: -4500. Both quadruples (Q1 and Q3) were set to unit resolution. Analyst software (version 1.4.2) was used to control sample acquisition and data analysis. To maximize sensitivity, ABA, SA, IAA, IAA-Asp, OPDA, JA standard solutions were infused into the 4000 QTRAP with a syringe pump (Harvard 22) at 10 mL min^{-1} to select multiple reaction monitoring (MRM) transitions and optimize compound-dependent parameters for MRM detection (Table 5.1). Because ion suppression was seen for these samples and obviated detection of IAA, an alternate high sensitivity method specific for IAA was used. This method uses the same column set-up, and the gradient was from 60% solvent A (0.1% (v:v) acetic acid in

Table 5.1. Optimized compound-dependent mass spectrometry parameters[a]

Compound	Q1	Q3	DP	EP	CE	CXP
SA	137	93	-49	-22	-5	-5
ABA	263.1	153	-60	-16.7	-9	-9
JA	209	59	-60	-24	-2	-2
H2JA	211	59	-60	-24	-2	-2
D4SA	142	98	-49	-22	-7	-7
OPDA	291.1	165	-75	-30	-5	-5
JA-ILE	322.1	130	-65	-32	-7	-7
D6ABA	269.1	159	-70	-16	-13	-13
IAA	174	130	-55	-16	-5	-5
D5IAA	179	135	-55	-16	-5	-5
IAA-ASP	289.1	88	-60	-32	-5.5	-5.5

[a]D6ABA was used as the internal standard for ABA, D4SA for SA, H2JA for JA and OPDA, D5IAA for IAA. Abbreviations of the compound dependent parameters are as follows: Q1, selected m/z of the first quadruple; Q3, selected m/z of the third quadruple; DT, dwell time monitoring each MRM transition (ms); DP, declustering potential of TIS source (V); CE, collision energy (arbitrary unit); CXP, collision cell exit potential (V); EP, collision cell exit potential (V).

MilliQ water), held for 2 min, to 100% solvent B (methanol with 0.1% acetic acid (v:v) in 4 min, and held for 5 min. The LC was then ramped back to initial conditions and re-equilibrated for 3 min. MRM transitions were 176/130 for IAA and 181/134 for D5IAA; DP = 55 volts, EP = 3.5 volts, CE = 24.5 volts, and CXP = 10 volts. The extraction efficiency of the internal standards was > 80%.

Hormone quantification

A standard curve was established for both methods. For quantification, a series of standards were prepared containing different concentrations of ABA, SA, IAA, IAA-Asp, OPDA, and JA mixed with the H_2JA and D-labeled ABA, SA, IAA (250 pmol/sample). Correction factors were obtained by adjusting the ratio of standard peak areas to that of internal standards in all samples. The peak areas of endogenous hormones were normalized with the corresponding internal standard and then calculated according to the standard curve. H_2JA was also used for the quantification of JA-Ile because no D-standard for that compound is commercially available. When IAA-Asp was detected, only the peak areas were reported because no D-standard is available for quantification and because ion suppression for this compound is difficult to accurately assess for absolute quantitation. However, the relative ratios of IAA-Asp across samples can be used for evaluating relative changes in the samples.

5.7 Acknowledgements

We thank ABRC for the T-DNA insertion lines, Dr. S-Y. Chen for providing the 35S:*ANAC092* seeds (Chinese Academy of Sciences, China), Dr. B. Zhang, Dr. S. Alvarez, and Dr. L. Hicks for their help with the samples sent to the Donald Danforth

Plant Science Center, and L. Zhenjiu for running the phytohormone analysis at the Donald Danforth Plant Science Center (St. Louise, Missouri, USA). We also thank the Dr. Shearer at PBI for submitting samples for analysis and the staff at PBI for performing the analysis.

Chapter 6:

Does age-related resistance include a developmentally-regulated priming process?

6.1 Preface

Chapter 6 was written by F. Al-Daoud and some ideas were contributed by Dr. R.K. Cameron.

6.2 Thesis summary

As Arabidopsis (*Arabidopsis thaliana*) ages it becomes more resistant to virulent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). Six week-old plants support up to 100-fold less *Pst* than younger plants (3 to 5 wpg: Kus et al., 2002). This is known as age-related resistance (ARR). ARR requires salicylic acid (SA) accumulation in the intercellular space and it is associated with flowering (Kus et al., 2002; Cameron and Zaton, 2004; Rusterucci et al., 2005). This thesis characterized age-related resistance (ARR) to *Pst* in Arabidopsis by exploring the role of two No Apical Meristem Cup-shaped Cotyledon (NAC) transcription factors: ANAC055 and ANAC092.

6.2a ANAC055 and ANAC092 play non-redundant roles in an EIN2-dependent pathway during ARR

A microarray experiment was conducted to identify genes that are differentially expressed during ARR in mature plants (6 wpg) inoculated with *Pst* compared to plants that were mock-inoculated (Chapter 2). Expression of a number of jasmonic acid/ethylene (JA/ET)-associated genes was up-regulated in this microarray, including two *NAC* transcription factors: *ANAC055* and *ANAC092*. To determine if the NACs are required for ARR, *anac055* and *anac092* T-DNA insertion mutants were tested for ARR. Mature *anac055* and *anac092* exhibit a modest increase in susceptibility to *Pst*, suggesting that *anac055* and *anac092* display a partial ARR defect (Chapter 2).

Therefore, ANAC055 and ANAC092 contribute to ARR. The partial ARR defect in *anac055* and *anac092* may be the result of partial redundancy between ANAC055 and ANAC092 during ARR. To test this idea, *anac055anac092* double mutants were produced and included in ARR experiments. Mature *anac055anac092* displays similar levels of susceptibility to *Pst* compared to *anac055* and *anac092* (Chapter 3), suggesting that the NACs play non-redundant roles in ARR. Alternatively, other NACs that are partially redundant with ANAC055 and/or ANAC092 may be contributing to the ARR phenotype of *anac055anac092*. For example, the third *NAC* gene that was up-regulated in the microarray, *ANAC029* (Chapter 2), may encode a protein that is partially redundant with ANAC055 and/or ANAC092. In addition, other NACs that are more similar to ANAC055 (such as ANAC019) and ANAC092 (such as ANAC059) may play partially redundant roles during ARR (Chapter 3). Preliminary data suggests that ANAC029 is not required for ARR, as mature *anac029* exhibits similar levels of resistance to *Pst* as the wild-type (Chapter 4).

ANAC055 and ANAC092 are functional transcription factors involved in JA/ET signaling in young plants during some development processes and in response to some biotic and abiotic stresses (Tran et al., 2004; He et al., 2005; Bu et al., 2008; Wang et al., 2008). Therefore, the NACs may also regulate JA/ET-associated gene expression during ARR. The ARR defect of *anac055* is associated with attenuated expression of some JA/ET-related genes, including *Lipoxygenase2* (*LOX2*: JA biosynthesis gene) and *Plant Defensin1.2a* (*PDF1.2a*: JA/ET signaling marker), and *Isochorismate Synthase1* (*ICS1*: SA biosynthesis gene) (Chapter 3). This suggests that ANAC055 is a positive regulator of

LOX2, *PDF1.2a*, and *ICS1* expression during ARR. ANAC055 may regulate these genes in a direct or indirect manner.

Unlike *anac055*, the ARR defect of *anac092* is associated with delayed flowering as fewer *anac092* plants possess visible inflorescence stems than Col-0 (at 6 wpg; Chapter 3). Since the onset of ARR is associated with flowering (Rusterucci et al., 2005), ARR assays were performed at different weeks to determine if the delayed flowering phenotype of *anac092* is associated with delayed onset of ARR. *anac092* is more susceptible to *Pst* at six wpg, whereas at seven wpg it is more resistant to *Pst* than the wild-type (Chapter 3). As a whole, this data illustrates that *anac092* exhibits an ARR defect at six wpg and an enhanced ARR response at seven wpg, and this is associated with a delay in flowering. The late flowering phenotype of *anac092* may be delaying the onset of ARR, which may be resulting in the ARR defect observed at six wpg and the enhanced ARR response observed at seven wpg. Furthermore, *ANAC092*-overexpressing plants (35S:*ANAC092*) are compromised in ARR, such that mature, six week-old 35S:*ANAC092* plants are modestly more susceptible to *Pst* than the wild-type. In addition, the ARR defect of mature 35S:*ANAC092* is associated with decreased expression of the JA/ET-associated genes *LOX2* and *PDF1.2a*, suggesting that ANAC092 is a negative regulator of *LOX2* and *PDF1.2a* expression during ARR (Chapter 3). Collectively, these data suggest that ANAC092 contributes to negative regulation of the ARR pathway, and it is a positive regulator of the onset of flowering and ARR. Furthermore, overexpressing *ANAC092* seems to induce early flowering, however, it does not appear to be sufficient to induce early onset of ARR. This is

illustrated by data that shows that 35S:*ANAC092* flowers earlier than the wild-type (35S:*ANAC092* possess more visible inflorescence stems than Col-0 at 4, 5, and 6 wpg; Chapter 6), but they exhibit wild-type levels of susceptibility to *Pst* in young plants, suggesting that they do not display ARR at a younger age.

The ARR defects of 35S:*ANAC092* and *anac055* are associated with attenuated expression of *LOX2* and *PDF1.2a*, and *anac055* also has decreased *ICS1* expression (Chapter 3). This suggests that mature 35S:*ANAC092* and *anac055* may have lower JA levels than the wild-type, and that *anac055* may accumulate less SA than mature wild-type plants after inoculation with *Pst*. To determine if the ARR defects of *anac055* and 35S:*ANAC092* are associated with altered phytohormone levels, free JA, JA-Ile (JA-isoleucine conjugate), 12-oxo-phytodienoic acid (OPDA: JA precursor), free SA, indole-3-acetic acid (IAA/auxin), IAA-Asp (IAA-aspartic acid conjugate), and abscisic acid (ABA) levels were quantified in mature wild-type plants, *anac055*, and 35S:*ANAC092* after inoculation with *Pst* (Chapter 5). Preliminary data illustrates that the ARR defects of *anac055* and 35S:*ANAC092* are associated with wild-type levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA suggesting that ANAC055 and ANAC092 doe not regulate accumulation of these phytohormones. These conclusions should be considered with caution as they are based on preliminary phytohormone analysis. Furthermore, other derivatives of JA, including methyl-JA (MeJA), were not quantified in these assays. MeJA and other JA derivatives play a role in JA/ET signaling (Kazan and Manners, 2008). The reduction in expression of *LOX2* and *PDF1.2a* that is associated with the

ARR defects of *anac055* and 35S:*ANAC092* may be the result of decreased accumulation of MeJA and/or other JA derivatives.

Expression of *ANAC055* and *ANAC092* is dependent on Ethylene Insensitive2 (EIN2), a central component of JA/ET signaling, in young plants responding to *Pseudomonas* (Wang et al., 2008). To determine how expression of the *NAC*s is regulated during ARR, *ANAC055* and *ANAC092* expression was observed in a number of JA/ET and SA signaling mutants, and in response to *Pst* strains that are defective in producing the JA/ET mimic coronatine. EIN2 seems to be a positive regulator of *ANAC055* and *ANAC092* expression during ARR, as expression of the *NAC*s is reduced in mature *ein2-1* compared to the wild-type in response to *Pst* (Chapter 3). Furthermore, expression of the *NAC*s is elevated in *lox2* and *ics1/sid2* compared to wild-type plants during ARR, suggesting that *ANAC055* and *ANAC092* expression is negatively regulated by LOX2-dependent JA signaling and ICS1-dependent SA signaling (Chapter 4). In addition, preliminary data suggests that *ANAC092* expression is elevated in mature *coronatine insensitve1* (*coi1*: a JA signaling mutant) compared to wild-type plants after inoculation with *Pst* (Chapter 4), suggesting that COI1 is involved in negative regulation of *ANAC092* expression. Also, *Pst*-produced coronatine does not seem to regulate *NAC* expression, as preliminary data illustrated that *ANAC055* and *ANAC092* are expressed to similar levels in mature plants inoculated with coronatine-deficient *Pst* compared to wild-type *Pst* (Chapter 4). As a whole, this data suggests that expression of *ANAC055* and *ANAC092* is positively regulated by EIN2 and negatively regulated by LOX2-dependent JA signaling and ICS1-dependent SA signaling. The preliminary nature of the coronatine

data, and the fact that expression of *ANAC055* and *ANAC092* in young plants appears to be at least partly regulated by *Pst* effectors (Truman et al., 2006; de Torres-Zabala et al., 2007) do not exclude the possibility that expression of *ANAC055* and *ANAC092* is regulated by *Pst* and/or the plant during ARR.

The gene expression data presented here suggests that expression of *ANAC055* and *ANAC092* is positively regulated by EIN2 and negatively regulated by LOX2-dependent JA signaling and ICS1-dependent SA signaling during ARR. However, the *NAC*s have different expression patterns during ARR compared to young plants after inoculation with *Pst*. *ANAC055* expression is reduced in mature compared to young plants after inoculation with *Pst*, whereas *ANAC092* is expressed earlier during ARR in mature plants compared to young plants after inoculation with *Pst* (Chapter 2). Furthermore, *ANAC092* is expressed early during ARR (at 5 min post inoculation, 12, 24, and 48 hpi), while *ANAC055* is expressed later during ARR (24 and 48 hpi: Chapter 2). Therefore, although *ANAC055* and *ANAC092* seem to be positively regulated by EIN2 and negatively regulated by LOX2-dependent JA and ICS1-dependent SA signaling, other signaling pathways may also regulate expression of *ANAC055* and *ANAC092* independently.

6.2b The relationship between flowering and ARR

Arabidopsis grown continuously under long days flower earlier and exhibit ARR earlier than plants grown under continuous short day conditions, suggesting that ARR is associated with flowering (Rusterucci et al., 2005). Plants grown under long days have an accelerated rate of development compared to plants grown under short days, therefore the

early onset of ARR under long days may be a result of accelerated development as a whole, and may not be necessarily caused by accelerated flowering. Since Arabidopsis is a facultative long day plant, early flowering can be induced in young plants grown under short days via the photoperiod pathway by exposing them to at least three long days (Koorneef et al., 1998; Levy and Dean, 1998; Simpson and Dean, 2002; Boss et al., 2004). This system allowed us to determine if inducing flowering in young, ARR-incompetent plants is sufficient to induce ARR. Short day-grown young Arabidopsis were exposed to at least three long days to induce flowering and they were included in ARR assays with control plants grown under continuous short days (Chapter 3). Long day-exposed young plants are more resistant to *Pst* than short day-grown young plants, suggesting that inducing flowering in young plants is associated with an ARR-like response. However, whether the onset of this ARR-like response occurs before, after, or is simultaneous with the initiation of flowering is a question that was not addressed in this study. This can be determined by performing daily ARR experiments after exposing Arabidopsis to long days to determine when the onset of the ARR-like response occurs. This should be accompanied by daily collection of plant samples to analyze expression of flowering marker genes to determine when the initiation of flowering occurs relative to the onset of the ARR-like response. Furthermore, how similar the ARR-like response is to ARR is also not known. To determine if the ARR-like response requires proteins that are also involved in ARR, ARR-defective mutants, such as *sid2*, can be included in future assays.

The ARR defect exhibited by *anac092* is associated with delayed flowering (Chapter 3), suggesting that ANAC092 is a positive regulator of flowering and the onset of ARR. To determine if the ARR defect of *anac092* is associated with altered phytohormone levels, JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA levels were quantified in mature *anac092* and wild-type plants after inoculation with *Pst*. Preliminary analysis revealed that the ARR defect of *anac092* is not associated with altered levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, or ABA (Chapter 5), suggesting that ANAC092 regulates the onset of ARR via a mechanism that is independent or downstream of these phytohormones. *ANAC092* is expressed in young plants in response to treatment with ET (Alonso et al., 2003), and it is expressed in an EIN2-dependent manner in young plants in response to some biotic (Wang et al., 2008) and abiotic stresses (He et al., 2005), during leaf senescence (Kim et al., 2009), and during ARR (Chapter 3). This suggests that the role of ANAC092 in flowering may involve regulating ET signaling. Therefore, the ET flowering pathway may regulate the onset of ARR. The role of ET in regulating flowering time is not well understood (Lin et al., 2009). Furthermore, there are three putative LEAFY binding sites in the promoter region of *ANAC092* (Chapter 2). LEAFY is a transcription factor that plays a role in the four main flowering pathways discussed below (Lee et al., 2006). This suggests that ANAC092 might regulate the onset of ARR by initiating flowering downstream of LEAFY. Other studies on plants that have a mutated *ANAC092* (ethyl methanesulphonate (EMS)-mutagenized *oresara 1-1* (*ore1-1*)) have shown that these plants are also delayed in leaf senescence (under long days; Kim et

al., 2009). Collectively, this data suggests that ANAC092 is a regulator of a number of plant development processes.

There are four main pathways that regulate flowering in Arabidopsis. The vernalization pathway initiates flowering in response to low temperatures. The photoperiod pathway initiates flowering when plants are exposed to long days. The gibberellin (GA) pathway initiates flowering under short days, and the autonomous pathway initiates flowering under short and long days (Koorneef et al., 1998; Lee et al., 2006; Levy and Dean, 1998; Simpson and Dean, 2002). To determine if the autonomous pathway regulates the onset of ARR, *luminidependens1* (*ld-1*), a late flowering mutant in the autonomous pathway (Redei, 1962; Lee et al., 1994; Aukerman et al., 1999; Domagalska et al., 2007), was tested for ARR (Chapter 3). Mature, six week-old *ld-1* displays a modest ARR defect, as it is four-fold more susceptible to *Pst* than the wild-type, suggesting that LD contributes to ARR. Therefore, the autonomous pathway appears to regulate the onset of ARR under short day conditions. Alternatively, LD may regulate ARR and flowering independently. In addition, the modest ARR defect in *ld-1* suggests that other flowering pathways may also regulate the onset of ARR, or other developmental ques (such as plant age) may be required for the onset of ARR. The idea that activation of a number of flowering pathways is required for the onset of ARR is supported by data that suggests that overexpressing *ANAC092* seems to be sufficient to induce early flowering, but it does not appear to induce early onset of ARR. Therefore, activating the ANAC092 flowering pathway may not lead to the onset of ARR because activation of other flowering pathways may be required.

We are only beginning to understand the relationship between flowering and ARR. The expression of flowering marker genes should be examined in plants exhibiting ARR to determine when the onset of ARR occurs relative to the initiation of flowering. Also, whether the GA and/or the vernalization pathways are involved in regulating the onset of ARR has not been tested. This can be done by determining if exposing Arabidopsis to GA or low temperatures (during vernalization studies plants are usually grown at 4 °C for up to 40 days) is associated with the onset of ARR, and by testing the ARR phenotypes of mutants in these pathways.

6.2c The role of JA during ARR

Expression of a number of JA/ET-associated genes was up-regulated in the ARR microarray presented in Chapter 2, suggesting that JA/ET signaling is involved in the ARR response. To test this hypothesis, expression of the JA/ET signaling marker *PDF1.2a* was monitored in young and mature plants after inoculation with *Pst*. *PDF1.2a* is expressed earlier and to elevated levels in mature compared to young plants after inoculation with *Pst* (Chapter 3). This is consistent with the idea that JA/ET signaling is up-regulated during ARR compared to young plants after inoculation with *Pst*. To determine if this apparent increase in JA/ET signaling during ARR is associated with increased JA production, JA, JA-Ile, and OPDA levels were quantified in young and mature plants after inoculation with *Pst*. JA, JA-Ile, and OPDA accumulate to similar levels in young and mature plants after inoculation with *Pst* (Chapter 5). In addition, a number of gene products that play a role in JA biosynthesis, such as LOX2, and JA signaling, such as JIN1 and JAR1, are not required for ARR (Chapter 3; Carviel et al.,

2009). Furthermore, preliminary data illustrates that the JA signaling mutant *coi1* and the JA biosynthesis mutant *opr3* exhibit enhanced ARR (Chapter 4), as mature *coi1* and *opr3* are more resistant to *Pst* than the wild-type. This suggests that some components of JA signaling negatively regulate the ARR pathway. This supports previously discussed data that suggested that expression of the *NAC*s is negatively regulated by LOX2-dependent JA signaling (Chapter 4). However, some JA signaling-associated genes, including *ANAC055* and *EIN2*, are required for ARR (Chapter 2 and Chapter 3). Collectively, this data suggests that JA signaling is involved in positive and negative regulation of ARR.

COI1 is a component of the ubiquitin E3 ligase SCF^{COI1} complex that promotes degradation of repressors of JA signaling, including members of the JAZ family, by ubiquitinating them and thereby directing them to the proteasome for degradation (Chini et al., 2007; Yan et al., 2007). COI1 could contribute to negative regulation of ARR by promoting degradation of positive regulators of ARR, such as transcriptional activators or transcription factors that are required for ARR gene expression. OPR3 plays a role in converting the JA precursor OPDA to JA (Sanders et al., 2000; Stinzi and Browse, 2000). OPR3 could play a role in repressing ARR by contributing to JA biosynthesis, which would induce downstream JA signaling including COI1-dependent pathways that inhibit ARR. Therefore, OPR3 and COI1 may negatively regulate ARR by inducing a JA signaling pathway that inhibits ARR.

The phytohormone analyses presented in this thesis did not quantify levels of some JA derivatives, including MeJA. MeJA and other JA derivatives play a role in JA/ET signaling (Kazan and Manners, 2008). Mature plants exhibiting ARR may

accumulate higher levels of MeJA and/or other JA derivatives than young plants after inoculation with *Pst*, and this could cause the observed increase in expression of some JA/ET-associated genes during ARR. Up-regulation of some JA/ET-associated genes in mature compared to young plants after inoculation with *Pst* may also be a result of hypersensitivity to JA and/or JA-related hormones. In other words, although mature plants do not seem to accumulate more JA, JA-Ile, or OPDA than young plants after inoculation with *Pst*, they may be more sensitive to these phytohormones. Hypersensitivity to JA, JA-Ile, and/or OPDA in mature plants may cause higher expression of some JA/ET-associated genes in mature plants than in young plants. Alternatively, this apparent increase in JA/ET signaling during ARR may be indicative of increased ET production and signaling in mature compared to young plants after inoculation with *Pst*. A synergistic interaction between ET and JA may occur during ARR and this may result in up-regulation of some JA/ET signaling. Also, *Pst* may produce more coronatine, a JA mimic, in mature than in young plants, and this might contribute to increased expression of some JA/ET-associated genes during ARR.

The ARR phenotypes of the JA mutants discussed in this thesis should be considered with caution, since some of them, such as *lox2* and *coi1*, have been shown to accumulate elevated levels of SA compared to wild-type plants after infection with pathogens (Kloek et al., 2001; Prithiviraj et al., 2005). Infiltration of SA into leaves of some ARR mutants partially rescues their ARR-defective phenotypes (Cameron and Zaton, 2004; Carviel et al., 2009). Therefore, hyperaccumulation of SA in JA mutants

might compensate for the effects of decreased JA levels on ARR, if any, and contribute to their wild-type ARR phenotypes.

6.2d SA plays a number of roles during ARR

Previous research in our lab suggests that SA is required for ARR (Kus et al., 2002; Cameron and Zaton, 2004; Carviel et al., 2009). However, SA does not appear to play its traditional role in up-regulating *Pathogenesis Related* (*PR*) gene expression, as the SA signaling mutant *non-expressor of PR genes1* (*npr1*) exhibits ARR and *PR1* expression is reduced in mature compared to young plants after 24 hpi (Kus et al., 2002; Rusterucci et al., 2005). SA appears to play an anti-microbial role in the intercellular space of plants exhibiting ARR (Cameron and Zaton, 2004).

The defense gene *PDF1.2a* is expressed earlier and to elevated levels during ARR compared to young plants after inoculation with *Pst* (Chapter 3), suggesting that other defense genes, including *PR1*, might also be expressed to elevated levels during ARR. To determine if *PR1* expression is elevated before 24 hpi during ARR, expression of *PR1* was analyzed in young and mature plants at early (before 24 hpi) and late (after 24 hpi) time points after inoculation with *Pst*. Preliminary data suggests that *PR1* expression is elevated during early stages of ARR and, as reported elsewhere (Kus et al., 2002; Rusterucci et al., 2005), it is expressed to lower levels during later stages of ARR compared to young plants after inoculation with *Pst* (Chapter 5). This suggests that *PR1* expression is elevated during early stages of ARR (before 24 hpi) compared to young plants after inoculation with *Pst*. NPR1 is not required for ARR (Kus et al., 2002), suggesting that expression of *PR1* during ARR is independent of NPR1. This *PR1*

expression pattern might be associated with elevated levels of SA during ARR. To test this idea, free SA was quantified in young and mature plants at early and late time points after inoculation with *Pst* (Chapter 5). SA appears to accumulate to similar levels in young and mature plants after *Pst* inoculation, suggesting that *PR1* expression is not associated with elevated SA levels during ARR. Expression of *PR1* during early stages of ARR (preliminary data) may be a result of hypersensitivity to SA. In other words, although mature plants do not seem to accumulate more SA than young plants after inoculation with *Pst*, they may be more sensitive to SA and initiate some SA signaling earlier than young plants. Alternatively, the expression pattern of *PR1* during ARR (preliminary data) may be associated with increased production of (an)other phytohormone(s).

The SA analyses presented in this thesis are not comprehensive. Free SA was quantified in the entire leaf without making any distinction between inter- and intracellular SA. Preliminary data shows that the ARR defects of *anac055* and 35S:*ANAC092* are not associated with a decrease in free SA accumulation when whole leaves are analyzed (Chapter 5). This does not excluded the possibility that *anac055* and 35S:*ANAC092* may have reduced levels of intercellular SA. Also, SA derivatives were not measured in these assays. Some SA derivatives, such as SA-glucoside (SAG), play important roles in SA storage (Vlot et al., 2009). SAG production may influence the level of free SA in plants. For example, production of SAG, by conjugating a glucose molecule to free SA, might result in lower amounts of free SA. Other SA derivatives, such as gentisic acid (2,5-dihydroxybenzoic acid), contribute to some defense signaling (Vlot et

al., 2009). For example, treating tomato plants with gentisic acid was found to induce accumulation of some PR proteins (Belles et al., 1999). Increased accumulation of some SA derivatives in plants exhibiting ARR compared to young plants after inoculation with *Pst* may contribute to expression of *PR1* during ARR.

6.3 Is early defense gene expression during ARR associated with increased ET production and signaling?

Expression of some JA/ET-associated genes is elevated in mature plants exhibiting ARR compared to young ARR-incompetent plants (Chapter 2; Chapter 3). However, young and mature plants have similar levels of JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA (Chapter 5). This suggests that increased production of (an)other phytohormone(s), such as ET, in mature plants might be contributing to the increased JA/ET gene expression during ARR. The idea that ET contributes to ARR signaling is consistent with a number of observations. Putative RAV1 binding sites are present in the promoter regions of *ANAC055* and *ANAC092* (Chapter 2). RAV1 belongs to the APETELLA2/Ethylene-Responsive Factor (AP2/ERF) superfamily (Kagaya et al., 1999) which regulates a number of JA/ET-associated defense responses downstream of EIN2 (Lorenzo et al., 2003; Gutterson and Reuber, 2004). The possibility that RAV1 regulates *ANAC055* and *ANAC092* during ARR is consistent with the EIN2-dependent expression of the *NAC*s (Chapter 3). EIN2 is a regulator of JA and ET signaling (Alonso et al., 1999). Furthermore, gene expression data demonstrates that *PDF1.2a* and *ANAC092* have a similar expression pattern during ARR, as both are expressed at 5 min, 12, 24, and 48 hpi (Chapter 2, Chapter 3). This suggests that they are co-regulated during ARR.

PDF1.2a is responsive to JA and ET (Schenk et al., 2000), and *ANAC092* is responsive to ET (Alonso et al., 2003) in young plants. Co-expression of *PDF1.2a* and *ANAC092* during ARR may be due to their common ET-responsive nature. This suggests that ET production increases in mature plants compared to young plants after inoculation with *Pst* (after 24 hpi).

Increased ET production during ARR could also explain the preliminary data that suggests that elevated expression of *PR1* occurs during early stages of ARR compared to young plants after inoculation with *Pst* (before 24 hpi; Chapter 5). ET augments SA-induced *PR1* expression, such that exposure of Arabidopsis to SA and ACC (1-aminocyclopropane-1-carboxylic acid), a precursor of ET, or SA and ET induce greater *PR1* expression than exposure to SA, ET, or ACC alone (Lawton et al., 1994; De Vos et al., 2006). Also, there are a number of studies that report increased resistance to *Pseudomonas* and increased *PR1* expression after ET signaling is up-regulated in plants (tobacco, Park et al., 2001; tomato, Gu et al., 2002). In Chapter 5, we showed that young and mature plants have similar levels of SA before 24 hpi with *Pst*. Increased ET production in that time frame (before 24 hpi) in mature plants could result in augmented expression of *PDF1.2a* and *PR1* and contribute to ARR. However, *PR1* is not responsive to ACC or ET alone (Lawton et al., 1994; De Vos et al., 2006), suggesting that ET production without SA production would not result in ARR. This is consistent with the ARR defects observed in SA-deficient plants (Kus et al., 2002; Carviel et al., 2009).

6.4 Does ARR include an amplified PTI response that is part of a developmentally-regulated priming mechanism?

ARR is characterized by a 10- to 100-fold decrease in *Pst* growth in mature compared to young plants (Kus et al., 2002). It requires intercellular accumulation of SA (Cameron and Zaton, 2005; Carviel et al., 2009). It is also associated with augmented expression of some JA/ET-associated genes (Chapter 2; Chapter 3; Carviel et al., 2009) and expression of a number of defense genes, including *PDF1.2a* (Chapter 3) and *PR1* (Chapter 5; preliminary data) during early stages of ARR (before 24 hpi). A number of JA/ET-associated gene products, such as ANAC055, ANAC092, and EIN2, contribute to ARR (Chapter 2; Chapter 3; Carviel et al., 2009). Phytohormone analyses revealed that ARR is associated with similar levels of JA, OPDA and JA-Ile compared to young plants after inoculation with *Pst* (Chapter 5).

Based on the data provided in this thesis, it is tempting to hypothesize that mature Arabidopsis is able to respond to and/or detect *Pst* much faster than young Arabidopsis (Carviel et al., 2009). This may be the result of a developmentally-regulated priming mechanism (priming reviewed in Conrath et al., 2006). This priming process would allow mature plants to activate enhanced defense signaling compared to young plants. For example, mature plants could amplify the defense responses exhibited by young plants. In other words, PAMP (pathogen-associated molecular pattern)-triggered Immunity (PTI) signaling that is negatively regulated by *Pst* in young plants may be enhanced in mature plants. This might overwhelm the mechanisms that *Pst* uses to negatively regulate PTI, and result in increased PTI signaling and resistance to *Pst* in mature plants. Overall, this

idea suggests that ARR includes a developmentally-regulated priming process that allows mature plants to activate an augmented PTI response. This idea will be explored here by comparing data from this thesis that characterize mature plants exhibiting ARR and data from the literature that characterize young plants displaying amplified PTI responses that result from reduced inhibition by *Pst*.

Reducing inhibition of PTI by *Pst* in Arabidopsis can be done in a number of ways: Arabidopsis can be inoculated with mutant *Pst* strains that have a diminished ability to negatively regulate PTI, or Arabidopsis can be treated with purified *Pst* PAMPs. *Pst* DC3000 *hrcC* has a weakened ability to inhibit PTI because it is not able to deliver its effectors into plant cells due to a mutated type III secretion system (TTSS; Yuan and He, 1996). Inoculating young Arabidopsis with *hrcC* induces early expression of *PR1*, and *PDF1.2* (within 9 hpi: Tsuda et al., 2008; Wang et al., 2009). This is similar to the elevated *PR1* (preliminary data) and *PDF1.2* expression observed during early stages of ARR (before 24 hpi; Chapter 3; Chapter 5). Young plants inoculated with *hrcC* have similar levels of SA as young plants inoculated with wild-type *Pseudomonas* bacteria at 24 hpi (Wang et al., 2009). Similarly, young ARR-incompetent plants and mature plants exhibiting ARR have similar levels of SA at 24 hpi with *Pst* (Chapter 5). This data demonstrates some similarities between the response in young plants to *Pst hrcC*, which has a compromised ability to suppress plant defenses, and mature plants exhibiting ARR.

Treating Arabidopsis with PAMPs purified from *Pst*, such as flg22 and elf18, also initiates PTI that is not negatively regulated by *Pst* (Bent and Mackey, 2007). A number

of biochemical and molecular changes occur in young Arabidopsis in response to flg22 and elf18 treatment. Within 15 min of flg22 and elf18 application, reactive oxygen species (ROS) production occurs (Gomez-Gomez et al., 1999; Aslam et al., 2009). Calcium accumulates within 30 min after treatment with flg22 and efl18 (Aslam et al., 2009). ET is produced within 6 hrs of flg22 and elf18 treatment (Bauer et al., 2001; Felix et al., 2002; Kunze et al., 2004; Navarro et al., 2004; Zipfel et al., 2006), and SA is produced in association with increased *ICS1* expression within 24 hrs of flg22 treatment (Tsuda et al., 2008; Wang et al., 2009). Flg22 induces expression of SA- and JA/ET-associated genes, including *PR1*, *PR5*, and *PDF1.2* (within 12 hrs), and elf18 induces expression of *PR1* (within 12 hrs) (Gomez-Gomez et al., 1999; Denoux et al., 2008; Aslam et al., 2009). Interestingly, *PR1* has a similar expression pattern during ARR in mature plants (preliminary data) and in young plants responding to flg22, as it is highly expressed at 12 hrs followed by a decrease in its expression level under both circumstances (Chapter 5; Denoux et al., 2008; Aslam et al., 2009). Taken together, this data suggests that treatment of Arabidopsis with *Pst* PAMPs results in early defense gene expression in association with SA accumulation and early production of ET (Bauer et al., 2001; Felix et al., 2002; Kunze et al., 2004; Navarro et al., 2004; Zipfel et al., 2006; Tsuda et al., 2008; Wang et al., 2009). This is similar to what is hypothesized to occur during ARR (this chapter).

The FLS2 pathway may contribute to the augmented PTI response that is hypothesized to occur in mature plants. After perception of flg22, FLS2 initiates a signaling pathway that includes a kinase cascade and activation of Mitogen-activated

Protein Kinase3 (MPK3) and MPK6 (Garcia-Brugger et al., 2006). MPK3 and MPK6 activate SA signaling (Colcombet and Hirt, 2008), and they promote ET production via 1-aminocyclopropane-1-carboxylic acid synthase2 and 6 (ACS2 and ACS6) (Ren et al., 2006; Xu et al., 2008). MPK6 also activates the Ethylene Responsive Factor104 (ERF104) transcription factor and EIN2-dependent signaling, including *PDF1.2* expression (Takahashi et al., 2007; Bethke et al., 2009). Therefore, production of ET and subsequent activation of JA/ET and SA signaling in Arabidopsis plants after treatment with flg22 may be partly due to MPK3/6-dependent signaling. Our data suggests that EIN2 contributes to ARR and it positively regulates *ANAC055* and *ANAC092* expression during ARR (Chapter 3). Signaling components upstream of EIN2, such as MPK3/6, may also contribute to ARR. The enhanced PTI signaling hypothesized to occur during ARR may include the FLS2-MPK3/6-EIN2-ANAC055/092 pathway.

Carviel et al. (2009) argued that ARR is not a form of PTI because it provides resistance against two different pathogen types, bacteria (*Pst*) and oomycetes (*H. arabidopsidis*), which possess different PAMPs. However, *mpk6* displays increased susceptibility to virulent *Pst* and a modest increase in susceptibility to virulent *H. arabidopsidis* (Menke et al., 2004). This suggests that PTI signaling that occurs after inoculation of Arabidopsis with *Pst* and *H. arabidopsidis* converge, at least in part, at MPK6. Therefore, MPK3/6-dependent PTI signaling during ARR could contribute to resistance to *Pst* and *H. arabidopsidis*.

The data presented thus far illustrate many parallels between amplified PTI and ARR. Mature Arabidopsis may reduce pathogen-induced negative regulation of PTI by a

number of mechanisms, including deactivating pathogen effectors and/or a priming process that allows it to activate an enhanced PTI. The latter scenario could include a developmentally-regulated priming event that might allow Arabidopsis to activate amplified PTI and contribute to resistance against *Pst* and *H. arabidopsidis*. Some priming mechanisms in Arabidopsis have been shown to up-regulate components of PTI that include the MPK3/6 pathway that is effective against *Pst* and *H. arabidopsidis*. For example, MPK3 and MPK6 are implicated in a priming event that occurs after treatment of Arabidopsis with the SA analogue benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH: Beckers et al., 2009). Upon treatment with BTH, inactive (not phosphorylated) MPK3 and MPK6 proteins accumulate in Arabidopsis. During a subsequent infection with *Pst*, active (phosphorylated) MPK3 and MPK6 accumulate for a longer period of time in primed plants than in plants that were not pretreated with BTH. This is associated with a rapid defense response, including early defense gene expression, and increased resistance to *Pst* (Beckers et al., 2009). A similar priming event may occur in mature Arabidopsis that allow it to up-regulate MPK3/6 signaling for a longer period of time than young plants.

Pst injects effectors into Arabidopsis that negatively regulate the FLS2-MPK3/6 pathway. For example, AvrPto and AvrPtoB bind to FLS2 and negatively regulate downstream signaling (Gohre et al., 2008; Xiang et al., 2008), and HopAI1 inhibits MPK3 and MPK6 activity (Zhang et al., 2007). If mature plants are primed and accumulate more MPK3/6 than non-primed young plants, then *Pst* might not be able to negatively regulate MPK3/6-mediated signaling as effectively in mature plants as it does

in young plants. This might result in increased MPK3/6 signaling during ARR compared to young plants after inoculation with *Pst*. This could induce expression of components downstream of MPK3/6, including SA and ET production, *PR1* and *PDF1.2a* expression, and EIN2-dependent signaling (up-regulation of *ANAC055* and *ANAC092*). In summary, one difference between young and mature plants may be that young plants are not primed and their PTI pathway is negatively regulated by *Pst*, whereas mature plants are primed and are able to activate enhanced PTI signaling that is not as effectively inhibited by *Pst*. This developmentally-regulated priming event may include up-regulation of MPK3/6 signaling.

6.5 Future directions

A number of testable hypotheses are presented in thesis:

1) ANAC055 may be redundant with ANAC019, ANAC072, and/or ANAC047. ANAC092 may be redundant with ANAC059, ANAC079, and/or ANAC100. ANAC029 may be redundant with ANAC055 and/or ANAC092. These hypotheses can be tested by producing multiple *nac* knockout mutants and determining their ARR phenotypes.
2) JA may be a negative regulator of some components of ARR. This hypothesis can be tested by treating plants exhibiting ARR with JA at different time points after inoculation with *Pst* (or a JA derivative) and determining if they exhibit a reduced ARR response.
3) Increased JA/ET signaling during ARR may be associated with increased ET production. This hypothesis can be tested by comparing ET production in

young and mature plants after inoculation with *Pst*. In addition, determine if young ET-overproducing plants (ex. *eto1* and *eto3*, Dr. JJ Kieber, U of North Carolina) have an ARR-like response.

4) ARR may include an amplified PTI response. This hypothesis can be tested by determining if PTI mutants, including *fls2*, *mpk3*, and *mpk6*, exhibit an ARR defect. Also, determine if ARR is comparable to PTI that is not fully inhibited by *Pst* in young plants. This can be done by comparing ARR in mature plants with young plants treated with *Pst* PAMPs, such as flg22, and/or young plants inoculated with mutant *Pst* that have a weakened ability to negatively regulate PTI, such as *Pst hrcC*.
5) ARR-competent plants may be primed in a MPK3/6-dependent manner. This hypothesis can be tested by determining if untreated mature plants accumulate more MPK3 and/or MPK6 transcripts and/or proteins than untreated young plants.

6.6 Significance of this research

This research project contributed to the understanding of ARR in Arabidopsis by characterizing the role of regulators of JA/ET signaling during ARR, the role of regulators of the onset of ARR and flowering, and the phytohormone profiles (JA, JA-Ile, OPDA, SA, IAA, IAA-Asp, and ABA) of young and mature plants after inoculation with *Pst*. Understanding the mechanisms that govern changes in resistance to pathogens as plants age will improve our ability to reduce the amount of crops lost due to disease by

allowing us to formulate treatments for plant disease that are optimal for different ages of plants.

References

Aarts N, Metz M, Holub E, Staskawicz BJ, Daniels MJ, Parker JE (1998) Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. PNAS **95:** 10306-10311

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell **15:** 63-78

Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Hosokawa D, Shinozaki K (1997) Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. Plant Cell **9:**1859-1868

Abel S, Nguyen MD, Chow W, Theologis A (1995) *ASC4*, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*. J Biol Chem **270:** 19093-19099

Abreu ME, Munne-Bosch S (2009) Salicylic acid deficiency in *NahG* transgenic lines and *sid2* mutants increases seed yield in the annual plant *Arabidopsis thaliana*. J Exp Bot **60:** 1261-1271

Adie B, Chico JM, Rubio-Somoza I, Solano R (2007) Modulation of plant defenses by ethylene. J Plant Growth Regul **26:** 160-177

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M (1997) Genes involved in organ separation in *Arabidopsis*: an analysis of the *cup-shaped cotyledon* mutant. Plant Cell **9:** 841-857

Aida M, Ishida T, Tasaka M (1999) Shoot apical meristem and cotyledon formation

during *Arabidopsis* embryogenesis: interaction among the *CUP-SHAPED COTYLEDON* and *SHOOT MERISTEMLESS* genes. Development **126:** 1563-1570

Alfano JR, Collmer A (2004) Type III secretion system effector proteins: double agents in bacterial disease. Annu Rev Phytopathol **42:** 385-414

Ali B, Sabri AN, Ljung K, Hasnain S (2009) Auxin production by plant associated bacteria: impact on endogenous IAA content and growth of *Triticum aestivum* L. Letters in App Microbiol **48:** 542-547

Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR (1999) EIN2, a bifunctional Transducer of ethylene and stress responses in *Arabidopsis*. Science **284:** 2148-2152

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Kames M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653-657

Anderson JP, Badruzsaufari, Schenk PM, Manners JM, Desmond OJ, Ehlert C,

Maclean DJ, Ebert PR, Kazan K (2004) Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. Plant Cell. **16:** 3460-3479

Arondel V, Vergnolle C, Cantrel C, Kader J-C (2000) Lipid transfer proteins are encoded by a small multigene family in *Arabidopsis thaliana*. Plant Science **157:** 1-12

Asai T, Tena G, Plotnikova j, Willmann MR, Chiu W-L, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signaling cascade in *Arabidopsis* innate immunity. Nature **415:** 977-983

Aslam SN, Erbs G, Morrissey KL, Newman M-A, Chinchilla D, Boller T, Molinaro A, Jasckson RW, Cooper RM (2009) Microbe-associated molecular pattern (MAMP) signatures, synergy, size and charge: influences on perception or mobility and host defence responses. MPP **10:** 375-387

Audenaert K, De Meyer GB, Hofte MM (2002) Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. Plant Physiol **128:** 491-501

Aukerman MJ, Lee I, Weigel D, Amasino RM (1999) The Arabidopsis flowering-time gene *LUMINIDEPEDENS* is a expressed primarily in regions of cell proliferation and encodes a nuclear protein that regulates *LEAFY* expression. Plant J **18:** 195-203

Axtell MJ, Chisholm ST, Dahlbeck D, Staskawicz (2003) Genetic and molecular

evidence that the *Pseudomonas syringae* type III effector protein AvrRpt2 is a cysteines protease. Mol Microbiol **49:** 1537-1546

Axtell MJ, Staskawicz BJ (2003) Initiation of *RPS2*-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. Cell **112:** 369-377

Bari R, Jones JDG (2009) Role of plant hormones in plant defence responses. Plant Mol Biol **69:** 473-488

Bauer Z, Gomez-Gomez L, Boller T, Felix G (2001) Sensitivity of different ecotypes and mutants of *Arabidopsis thaliana* toward the bacterial elicitor flagellin correlates with the presence of receptor-binding sites. J Biol Chem **49:** 45669-45676

Beckers GJM, Jaskiewicz M, Liu Y, Underwook WR, He SY (2009) Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana*. Plant Cell **21:** 944-953

Bell E, Creelman RA, Mullet JE (1995) A chloroplast Lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. PNAS **92:** 8675-8679

Bell E, Mullet JE (1993) Characterization of an *Arabidopsis* lipoxygenase gene responsive to methyl jasmonate and wounding. Plant Physiol **103:** 1133-1137

Belles JM, Garro R, Fayos J, Navarro P, Primo J, Conejero V (1999) Gentisic acid as a pathogen-inducible signal, additional to salicylic acid for activation of plant defenses in tomato. MPMI **12:** 227-235

Bender CL, Alarcon-Chaidez F, Gross DC (1999) *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol Mol Biol Rev **63:** 266-292

Benschop JJ, Millenaar FF, Smeets ME, van Zanten M, Voesenek LACJ, Peeters AJM (2007) Abscisic acid antagonizes ethylene-induced hyponastic growth in Arabidopsis. Plant Physiol **143:** 1013-1023

Bent AF, Innes RW, Ecker JR, Staskawicz BJ (1992) Disease development in ethylene-Insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. MPMI **5:** 372-378

Bent AF, Mackey D (2007) Elicitors, effectors and *R* genes: the new paradigm and lifetime supply of questions. Annu Rev Phytopath **45:** 399-436

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

Berghammer H, Auer B (1993) Easypreps – fast and easy plasmid minipreparation for analysis of recombinant clones in *Escherichia coli*. Biotechniques **14:** 522-525

Berrocal-Lobo M, Molina A (2004) Ethylene Response Factor 1 mediates *Arabidopsis* resistance to the soilborne fungus *Fusarium oxysporum*. Mol Plant Mol Inter **17:** 763-770

Bethke G, Unthan T, Uhrig JF, Poschl Y, Gust AA, Scheel D, Lee J (2009) Flg22 regulates the release of an ethylene response factor substrate from MAP kinase 6 in *Arabidopsis thaliana* via ethylene signaling. PNAS **106:** 8067-8072

van der Biezen, Jones JDG (1998) Plant disease-resistance proteins and the gene-for-gene concept. TIBS **23:** 454-456

Boller T, He SY (2009) Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. Science **324:** 742-744

Boller T, Felix G (2009) Patterns and danger signals by pattern-recognitions receptors. Annu Rev Plant Biol **60:** 379-606

Brodersen P, Petersen M, Nielsen HB, Zhu S, Newman M-A, Shokat KM, Rietz S, Parker J, Mundy J (2006) Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. Plant J **47:** 532-546

Blanco F, Garreton V, Frey N, Dominguez C, Perez-Acle T, Van der Straeten D, Jordana X, Holuigue L (2005) Identification of NPR1-dependent and independent genes early induced by salicylic acid treatment in Arabidopsis. Plant Mol Biol **59:** 927-944

Blazquez MA, Soowal LN, Lee I, Weigel D (1997) LEAFY expression and flower initiation in Arabidopsis. Development **124:** 3835-3844

Bleeker AB, Estelle MA, Somerville C, Kende H (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science **241:** 1086-1089

Block A, Schmelz E, Jones JB, Klee HJ (2005) Coronatine and salicylic acid: the battle between Arabidopsis and *Pseudomonas* for phytohormone control. MPP **6:** 79-83

Boss PK, Bastow RM, Myine JS, Dean C (2004) Multiple pathways in the decision to Flower: enabling, promoting, and resetting. Plant Cell **16:** S18-S31

Brooks DM, Bender CL, Kunkel BN (2005) The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. MPP **6:** 629-639

Brown RL, Kazan K, McGrath KC, Maclean DJ, Manners JM (2003) A role for the GCC-box in jasmonate-mediated activation of the *PDF1.2* gene of Arabidopsis. Plant Physiol **132:** 1020-1032

Bu Q, Jiang H, Li C-B, Zhai Q, Zhang J, Wu X, Sun J, Xie Q, Li C (2008) Role of the *Arabidopsis thaliana* NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. Cell Research **18:** 756-767

Buhrman DL, Price PI, Rudewicz PJ (1996) Quantitation of SR 27417 in human plasma using electrospray liquid chromatography-tandem mass spectrometry: a study of ion suppression. J Am Soc Mass Sepctrom **7:** 1099-1105

Cameron RK, Paiva NL, Lamb CJ, Dixon RA (1999) Accumulation of salicylic acid and PR-1 gene transcripts in relation to the systemic acquired resistance (SAR) response induced by *Pseudomonas syringae* pv. *tomato* in *Arabidopsis*. PMPP **55:** 121-130

Cameron RK, Zaton K (2004) Intercellular salicylic acid accumulation is important for Age-Related Resistance in *Arabidopsis* to *Pseudomonas syringae*. PMPP **65:** 197-209

Canals J, Pinochet J (1992) Temperature and age of plant affect resistance in peach-

almond hybrid rootstock infected with *Meliodogyne javanica*. Horticultural Science **27**: 1211-1213

Cao H, Bowling SA, Gordon S, Dong X (1994) Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6:**1583-1592

Carviel JL, Al-Daoud F, Meumann M, Mohammad A, Provart NJ, Moeder W, Yoshioka K, Cameron RK (2009) Forward and reverse genetics to identify genes involved in the age-related resistance response in *Arabidopsis thaliana*. MPP **10:** 621-634

Cecchini E, Geri C, Love AJ, Coupland G, Covey SN, Milner JJ (2002) Mutations that delay flowering in *Arabidopsis* de-couple symptom response from cauliflower mosaic virus accumulation during infection. MPP **3:** 81-90

Century KS, Shapiro AD, Repetti PP, Dahlbeck D, Holub E, Staskawicz (1997) *NDR1*, a pathogen-induced component required for *Arabidopsis* disease resistance. **278:** 1963-1965

Champigny MJ, Cameron RK (2009) Action at a distance: long-distance signals in induced resistance *In* Advances in Botanical Research (editor: van Loon LC) **51:** 124-173

Chaturvedi R, Krothapalli K, Makandar R, Nandi A, Sparks AA, Roth MR, Welti R, Shah J (2008) Plastid omega 3-fatty acid desaturase-dependent accumulation of a systemic acquired resistance inducing activity in petiole exudates of *Arabidopsis thaliana* is independent of jasmonic acid. Plant J **54:** 106-117

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. Science **262:** 539-544

Chen C, Chen Z (2002) Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced *Arabidopsis* transcription factor. Plant Physiol **129**: 706-716

Chen H, Xue L, Chintamanani S, Germain H, Lin H, Cui H, Cai R, Zuo J, Tang X, Li X, Guo H, Zhou J-M (2009) ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE-LIKE1 repress *SALICYLIC ACID INDUCTION DEFICIENT2* expression to negatively regulate plant innate immunity in *Arabidopsis*. Plant Cell **21:** 2527-2540

Chen Q, Zhang B, Hicks LM, Zhang Q, Jez JM (2009) "A liquid chromatography-tandem mass spectrometry-based assay for indole-2-acetic acid-amido synthetases," Anal. Biochem. **390:** 149-154

Chen Z, Agnew JL, Cohen JD, He P, Shan L, Sheen J, Kunkel BN (2007) *Pseudomonas syringae* type III effector AvrRpt2 alters *Arabidopsis thaliana* auxin physiology. PNAS **104:** 20131-20136

Chen Z, Silva H, Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science **262:** 1883-1886

Chern M, Canlas PE, Fitzgerald HA, Ronald PC (2005) Rice NRR, a negative regulator of disease resistance, interacts with *Arabidopsis* NPR1 and rice NH1. Plant J **43**: 623-635

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nurnberger T, Jones JDG, Felix G, Boller T (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature **448:** 497-500

Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signaling. Nature **448:** 666-672

Chintamanani S, Multani DS, Ruess H, Johal GS (2008) Distinct mechanisms govern the dosage-dependent and developmentally regulated resistance conferred by the maize *Hm2* gene. MPMI **21:** 79-86

Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell **124:** 803-814

Choi SH, Gu MB (2001) Phenolic toxicity – detection and classification through the use of a recombinant bioluminescent *Escherichia coli*. Environmental Toxicology and Chemistry **20:** 248-255

Clark AM, Bohnert HJ (1999) Cell-specific expression of genes of the lipid transfer protein family from *Arabidopsis thaliana*. Plant Cell Physiol **40:** 69-76

Cohn JR, Martin GB (2005) *Pseudomonas syringae* pv. *tomato* type III effectors AvrPto and AvrPtoB promote ethylene-dependent cell death in tomato. Plant J **44:** 139-154

Colcombet J, Hirt H (2008) *Arabidopsis* MAPKs: a complex signaling network involved in multiple biological processes. Biochem J **413:** 217-226

Collinge M, Boller T (2001) Differential induction of two potato genes, *Stprx2*, and *StNAC*, in response to infection by *Phytophthora infestans* and to wounding. Plant Mol Biol **46:** 521-529

Conrath U, Beckers GJM, Flors V, García-Agustín P, Jakab G, Mauch F, Newman M-A, Pieterse CMJ, Poinssot B, Pozo MJ, Pugin A (2006) Priming: Getting Ready for Battle. MPMI **10:** 1062-1071

Corbesier L, Coupland G (2006) The quest for florigen: a review of recent progress. J Exp Botany **57:** 3395-3403

Crocoll C, Kettner J, Dorffling K (1991) Abscisic acid in saprophytic and parasitic species of fungi. Phytochemistry **4:** 1059-1060

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. Nature **411:** 826-833

Davuluri RV, Sun H, Palaniswamy SK, Matthews N, Molina C, Kurtz M, Grotewold E (2003) AGRIS: *Arabidopsis* gene regulatory information server, an information resource of *Arabidopsis cis*-regulatory elements and transcription factors. BMC Bioinformatics **4:**25

Day B, Dahlbeck D, Staskawicz BJ (2006) NDR1 interaction with RIN4 mediates the differential activation of multiple disease resistance pathways in *Arabidopsis*. Plant Cell **18:** 2782-2791

De Vos M, Van Oosten VR, Van Poecke RMP, Van Pelt JA, Pozo MJ, Mueller MJ,

Buchala AJ, Metraux JP, Van Loon LC, Dicke M, Pieterse MJ (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. MPMI **18:** 923-937

De Vos M, Van Zaanen W, Koornneef A, Korzelius JP, Dicke M, Van Loon LC, Pieterse MJC (2006) Herbivore-induced resistance against microbial pathogens in *Arabidopsis*. Plant Physiol **142:** 352-363

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weyman K, Negrotto D (1994) A central role for salicylic acid in plant disease resistance. Science **266**, 1247-50.

Delessert C, Kazan K, Wilson IW, van der Straeten D, Manners J, Dennis ES, Dolferus R (2005) The transcription factor ATAF2 represses the expression of pathogenesis-related genes in *Arabidopsis*. Plant J **43:** 745-757

Dempsey DA, Shah J, Klessig DF (1999) Salicylic acid and disease resistance in plants. Crit Rev Plant Sciences **18:** 547-575

Denoux C, Galletti R, Mammarella N, Gopalan S, Werck D, De Lorenzo G, Ferrari S, Ausubel FM, Dewdney J (2008) Activation of defense response pathways by OGs and flg22 elicitors in *Arabidopsis* seedlings. Mol Plant **1:** 423-445

Deslandes L, Olivier J, Peeters N, Feng DX, Khounlotham M, Boucher C, Somssich I, Genin, Marco Y (2003) Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. PNAS **100:** 8024-8029

Desveaux D, Subramaniam R, Despres C, Mess J-N, Levesque C, Fobert PR, Dangl

JL, Brisson N (2004) A "Whirly" transcription factor is required for salicylic acid-dependent disease resistance in *Arabidopsis*. Developmental Cell **6:** 229-240

Devadas SK, Enyedi A, Raina R (2002) The *Arabidopsis hrl1* mutation reveals novel overlapping roles for salicylic acid, jasmonic acid and ethylene signaling in cell death and defence against pathogens. Plant J **30:** 467-480

Develey-Riviere MP, Galiana E (2007) Resistance to pathogens and host developmental stage: a multifaceted relationship within the plant kingdom. New Phytologist **175:** 405-416

Devoto A, Ellis C, Magusin A, Chang H-S, Chilcott C, Zhu T, Turner JG (2005) Expression profiling reveals *COI1* to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. Plant Mol Biol **58:** 497-513

Dharmasiri N, Dharmasiri S, Estelle M (2005) The F-box protein TIR1 is an auxin receptor. Nature **435:** 441-445

Dickson MH, Petzoldt R (1993) Plant age and isolate source affect expression of downy mildew resistance in broccoli. Horticultural Science **28**: 730-731

DiFonzo CD, Ragsdale DW, Radcliffe EB, Banttari EE (1994) Susceptibility of potato leafroll virus in potato: effects of cultivar, plant age at inoculation, and inoculation pressure on tuber infection. Plant Disease **78**: 1173-1177

Domagalska MA, Schomburg FM, Amasino RM, Vierstra RD, Nagy F, Davis SJ (2007) Attenuation of brassinosteroid signaling enhances *FLC* expression and delays flowering. Development **134:** 2841-2850

Dombrecht B, Xue GP, Sprague SJ, Kirkegaard JA, Ross JJ, Reid JB, Fitt GP, Sewelam N, Schenck PM, Manners JM, Kazan K (2007) MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. Plant Cell **19:** 2225-2245

Dong X (2004) NPR1, all things considered. Current Opinion in Plant Biology **7:** 547-552

Dubey SC (1997) Influence of age of plants, temperature and humidity on web blight development in groundnut. Indian Phytopathol **50**: 119-120

Durrant WE, Dong X (2004) Systemic Acquired Resistance. Annu Rev Phytopathol **42:** 185-209

Duval M, Hsieh T, Kim SY, Thomas TL (2002) Molecular characterization of *AtNAM*: a member of the *Arabidopsis* NAC domain superfamily. Plant Mol. Biol. **50:** 237-248

Erbs G, Neman M-A (2003) The role of lipopolysaccharides in induction of plant defence responses. MPP **4:** 421-425

Ehlting J, Provart NJ, Werck-Reichhart D (2006) Functional annotation of the *Arabidopsis* P450 superfamily based on large-scale co-expression analysis. Biochem Society Transactions. **34:** 1192-1198

Eriksson S, Bohlenius H, Moritz T, Nilsson O (2006) GA_4 is the active gibberellin in the regulation of *LEAFY* transcription and *Arabidopsis* floral initiation. Plant Cell **18:** 2172-2181

Faivre-Nitschke SE, Grienenberger JM, Gualberto JM (1999) A prokaryotic-

type cytidine deaminase from *Arabidopsis thaliana* gene expression and functional characterization. Eur J Biochem **263:** 896-903

Falk A, Feys B, Frost LN, Jones JDG, Daniels MJ, Parker JE (1999) *EDS1*, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. PNAS **96:** 3292-3297

Farmer EE, Almeras E, Krishnamurthy (2003) Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr Opin Plant Biol **6:** 372-378

Felix G, Duran JD, Volko S, Boller T (2002) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. **18:** 265-276

Ferguson IB, Mitchell RE (1985) Stimulation of ethylene production in bean leaf discs by the Pseudomonad phytotoxin coronatine. Plant Physiol. **77:** 969-973

Feys BJ, Benedetti CE, Penfold CN, Turner JG (1994) Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell. **6:** 751-759

Feussner I, Wasternack C (2002) The Lipoxygenase pathway. Ann Rev Plant Biol **53:** 275-297

Feys FJ, Moisan LJ, Newman MA, Parker JE (2001) Direct interaction between the *Arabidopsis* disease resistance signaling proteins, *EDS1* and *PAD4*. EMBO **20:** 5400-5411

Flor HH (1942) Inheritance of pathogenicity in a cross between physiologic races 22 and 24 of *Melampsora lini*. Phytopathlogy **32:** 5

Flors V, Ton J, van Doorn R, Jakab G, Garcia-Agustin P, Mauch-Mani B (2008)

Interplay between JA, SA and ABA signaling during basal and induced resistance against *Pseudomonas syringae* and *Alternaria brassicicola*. Plant J **54:** 81-92

Flors V, Ton J, Jakab G, Mauchi-Mani B (2005) Abscisic acid and callose: team players in defence against pathogens. J Phytopath **153:** 377-383

Fobert P, Despres C, (2005) Redox control of systemic acquired resistance. Curr Opin Plant Biol **8:** 378-382

Forouhar F, Yang Y, Kumar D, Chen Y, Fridman E, Park SW, Chiang Y, Acton TB, Montelione GT, Pichersky E, Klessig DF, Tong L (2005) Structural and biochemical studies identify tobacco SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity. PNAS **102:** 1773-1778

Fouts DE, Abramovitch RB, Alfano JR, Baldo AM, Buell CR, Cartinhour S, Chatterjee AK, D'Ascenzo M, Gwinn ML, Lazarowitz SG, Lin N-C, Martin GB, Rehm AH, Schneider DJ, van Dijk K, Tang X, Collmer A (2002) Genomewide identification of *Pseudomonas syringae* pv. *tomato* DC3000 promoters controlled by the HrpL alternative sigma factor. PNAS **99:** 2275-2280

Fraser RSS (1981) Evidence for the occurrence of the pathogenesis-related proteins in leaves of healthy tobacco plants during flowering. Physiol Plant Pathol **19:** 69-76

Frye CA, Tang D, Innes RW (2001) Negative regulation of defense responses in plants by conserved MAPKK kinase. PNAS **98:** 373-378

Fuji H, Verslues PE, Zhu J-K (2007) Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in *Arabidopsis*. Plant Cell **19:** 485-494

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M (2000) *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell **12:** 393-404

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran P L-S, Yamaguchi-Shinozaki K, Shinozaki K (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. Plant J **39:** 863-876

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

Garcia-Brugger A, Lamotte O, Vandelle E, Bourque S, Lecourieux D, Poinssot B, Wendehenne D, Pugin A (2006) Early signaling events induced by elicitors of plant defenses. MPMI **19:** 711-724

Garcia-Olmedo F, Molina A, Segura A, Moreno M (1995) The defensive role of nonspecific lipid-transfer proteins in plants. Trends Microbiol **3:** 72-74

Giorcelli A, Vietto L, Anselmi N, Gennaro M (1996) Influence of clonal susceptibility, leaf age and inoculum density on infections by *Melampsora larici-populinai* races E1 and E3. Europ J Forest Pathol **26**: 323-331

Giuliano G, Pichersky E, Malik VS, Timko MP, Scolnik PA, Cashmore AR (1988) An evolutionary conserved protein binding sequence upstream of a plant light-regulated gene. PNAS **85:** 7089-7093.

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and

necrotrophic pathogens. Annu. Rev Phytopathol **43:** 9.1-9.23

Glazebrook J, Rogers EE, Ausubel FM (1996) Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. Genetics **143:** 973-982

Grant MR, Jones JDG (2009) Hormone (dis)harmony moulds plant health and disease. Science **324:** 750-752

Gray WM, Kepinski S, Rouse D, Leyser O, Estelle (2001) Auxin regulates SCF^{TIR1}-dependent degradation of AUX/IAA proteins. Nature **414:** 271-276

Gohre V, Spallek T, Haweker H, Mersmann S, Mentzel T, Boller T, de Torres M, Mansfield JW, Robatzek S (2008) Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. Current Biol **18:** 1824-1832

Gomez-Gomez L, Boller T (2000) FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol Cell **5:** 1003-1011

Gomez-Gomez L, Felix G, Boller T (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. Plant J **18:** 277-284

Goss EM, Bergelson J (2006) Variation in resistance and virulence in the interaction between *Arabidopsis thaliana* and a bacterial pathogen. Evolution **60:** 1562-1573

Gott JM, Emeson RB (2000) Functions and mechanisms of RNA editing. Ann Rev Genet **34:** 499-531

Guilfoyle T, Hagen G, Ulmasov T, Murfett J (1998) How does auxin turn on genes? Plant Physiol **118:** 341-347

Gu Y-Q, Wildermuth MC, Chakravarthy S, Loh Y-T, Yang C, He X, Han Y,

Martin GB (2002) Tomato transcription factors Pti4, Pti5, and Pti6 activate defense responses when expressed in *Arabidopsis*. Plant Cell **14:** 817-831

Guo H, Ecker JR (2003) Plant responses to ethylene gas are mediated by $SCF^{EBF1/EBF2}$-dependent proteolysis of EIN3 transcription factor. Cell **115:** 667-677

Guo H-S, Xie Q, Fei J-F, Chua N-H (2005) MicroRNA directs mRNA cleavage of the transcription factor *NAC1* to downregulate auxin signals for *Arabidopsis* lateral root development. Plant Cell. **17:** 1376-1386

Guo Y, Gan S (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J **46:** 601-612

Gutterson N, Reuber TL (2004) Regulation of disease resistance pathways by AP2/ERF transcription factors. Curr Opin Plant Biol **7:** 465-471

Guttman DS, Vinatzer BA, Sarkar SF, Ranall MV, Kettler G, Greenberg JT (2002) A functional screen for the Type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. Science **295:** 1722-1726

Hall AE, Bleecker AB (2003) Analysis of combinatorial loss-of-function mutants in the *Arabidopsis* ethylene receptors reveals that the *ers1etr1* double mutant has severe developmental defects that are EIN2 dependent. Plant Cell **15:** 2032-2041

Hansen H, Gorssmann K (2000) Auxin-induced ethylene triggers abscisic acid biosynthesis and growth inhibition. Plant Physiol **124:** 1437-1448

Hartman U, Hohmann S, Nettesheim K, Wisman E, Saedler H, Huijser P (2000) Molecular cloning of *SVP*: a negative regulator of the floral transition in *Arabidopsis*. Plant J. **21:** 351-360

Hauck P, Thilmony R, He SY (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. PNAS **100:** 8577-8582

He P, Chintamanani S, Chen Z, Zhu L, Kunkel BN, Alfano JR, Tang X, Zhou J-M (2004) Activation of a COI1-dependent pathway in *Arabidopsis* by *Pseudomonas syringae* type III effectors and coronatine. Plant J **37:** 589-602

He XJ, Mu RL, Cao WH, Zhang ZG, Zhang JS, Chen SY (2005) AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is Involved in salt stress response and lateral root development. Plant J **44:** 903-916

Heese A, Hann DR, Gimenez-Ibanez S, Jones AME, He K, Li J, Schroeder JI, Peck SC, Rathjen JP (2007) The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. PNAS **104:** 12217-12222

Hegedus D, Yu M, Baldwin D, Gruber M, Sharpe A, Parkin I, Whitwill S, Lydiate D (2003) Molecular characterization of *Brassica napus* NAC domain transcriptional activators in response to biotic and abiotic stress. Plant Mol. Biol. **53:** 383-397

Heitefuss R, Ebrahim-Nesbat F, Ordonez MT, and Schorn-Kasten K (1997) Investigations on adult plant resistance of barley against *Erysiphe graminis* f.sp. *hordei*. J Phytopath **145**: 177-184

Hellmann H, Estelle M (2002) Plant development: regulation by protein degradation. Science **297:** 793-797

Hellmann H, Hobbie L, Chapman A, Dharmasiri S, Dharmasiri N, del Pozo C,

Reinhardt D, Estelle M (2003) *Arabidopsis AXR6* encodes CUL1 implicating SCF E3 ligases in auxin regulation of embryogenesis. EMBO **22:** 3314-3325

Hennig J, Malamy J, Grynkiewicz G, Indulski J, Klessig DF (1993) Interconversion of the salicylic acid signal and its glucoside in tobacco. Plant J **4:** 593-600

Herbers K, Meuwly P, Metraux J-P, Sonnewald U (1996) Salicylic acid-independent induction of pathogenesis-related protein transcripts by sugars is dependent on leaf developmental stage. FEBS Letters. **397**: 239-244

Hibara K, Karim R, Takada S, Taoka K, Furutani M, Aida M, Tasaka M (2006) *Arabidopsis CUP-SHAPED COTYLEDON3* regulates postembryonic shoot meristem and organ boundary formation. Plant Cell **18:** 2946-2957

Hu H. Dai M, Yao J, Xiao B, Li X, Zhang Q, Xiong L (2006) Overexpression of NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. PNAS. **103:** 12987-12992

Hua J, Meyerowitz EM (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell **94:** 261-271

Hugot K, Aime S, Conrod S, Poupet A, Galiana E (1999) Developmental regulated mechanisms affect the ability of a fungal pathogen to infect and colonize tobacco leaves. Plant J **20**: 163-70

Hugot K, Riviere M-P, Moreilhon C, Dayem MA, Cozzitorto J, Arbiol G, Barbry P, Weiss C, Galiana E (2004) Coordinated regulation of genes for secretion in tobacco at late developmental stages: association with resistance against oomycetes. Plant Physiol **134:** 858-870

Hwang BK (1995) Effect of age-related resistance and metalaxyl on capsidiol production in pepper plants infected with *Phytophthora capsici*. *in* Handbook of Phytoalexin Metabolism and action. Eds. Daniel M, Purkayastha. Marcel Dekker Inc.

Huber SC, Hardin SC (2004) Numerous posttranslational modifications provide opportunities for the intricate regulation of metabolic enzymes at multiple levels. Curr Opin Plant Biol **7:** 318-322

Hugot K, Aime S, Conrod S, Poupet A, and Galiana E (1999) Developmental regulated mechanisms affect the ability of a fungal pathogen to infect and colonize tobacco leaves. Plant J **20**: 163-70.

Iyer LM, Koonin EV, Aravind L (2001) Adaptations of the helix-grip fold for ligand binding and catalysis in the START domain superfamily. Proteins **43:** 134-144

Johnson C, Lods L, Scholz D, Dres C, Dashnaw RA, Linstra L, Brooks G (2000) Maintaining the integrity of cosmetic formulations via the utilization of endogenous signal molecules from plants. Agro Food Industry Hi-Tech **11:** 23-24

Jones PR, Moller BL, Hoj PB (1999) The UDP-glucose:*p*-Hydroxymandelonitrile-*O*-Glucosyltransferase that catalyzes the last step in synthesis of the cyanogenic glucoside *Sorghum bicolor*. J Biol Chem **274:** 35483-35491

Journot-Catalino N, Somssich IE, Roby D, Kroj T (2006) The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in *Arabidopsis thaliana*. Plant Cell **16:** 3289-3302

Kagaya Y, Ohmiya K, Hattori T (1999) RAV1, a novel DNA-binding protein, binds to

bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants. Nuc Acids Res **27:** 470-478

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. PNAS **105:** 7100-7105

Kazan K, Manners JM (2008) Jasmonate signaling: toward an integrated view. Plant Phys **146:** 1459-1468

Kazan K, Manner JM (2009) Linking development to defense: auxin in plant-pathogen interactions. Trends Plant Science **14:** 373-382

Kepinski S, Leyser O (2005) The *Arabidopsis* F-box protein TIR1 is an auxin receptor. Nature **435:** 446-451

Kesarwani M, Yoo J, Dong X (2007) Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in Arabidopsis. Plant Physiol **144:** 336-346

Kettner J, Dorffling K (1995) Biosynthesis and metabolism of abscisic acid in tomato leaves infected with *Botrytis cinerea.* Planta **196:** 627-634

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (1993) *CTR1*, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. Cell **72:** 427-441

Kift NB, Dewar AM, Werker AR, Kixon AFG (1996) The effect of plant age and infection with virus yellows on the survival of *Myzus persicae* on sugar beet . Annuals of Applied Biology. **129**: 371-378

Kim JH, Woo HR, Kim J, Lim PO, Lee IC, Choi SH, Hwang D, Nam HG (2009) Trifurcate feed-forward regulation of age-dependent cell death involving *miR164* in *Arabidopsis*. Science **323:** 1053-1057

Kim MG, da Cunha L, McFall AJ, Belkhadir Y, DebRoy S, Dangl JL, Mackey D (2005) Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defense in *Arabidopsis*. Cell **121:** 749-759

Kim TW, Hwang JY, Kim YS, Joo SH, Chang SS, Lee JS, Takatsuto S, Kim SK (2005) *Arabidopsis* CYP85A2, a cytochrome P450, mediates the Baeyer-Villiger oxidation of castasterone to brassinolide in brassinosteroid biosynthesis. Plant Cell **17:** 2397-2412

Kim YH, Hwang BK, Park KW (1989) Expression of age-related resistance in pepper plants infected with *Phytophthora capsici*. Plant Disease **73**:745-747.

Kim YS, Kim SG, Park JE, Park HY, Lim MH, Chua NH, Park CM (2006) A membrane-bound *NAC* transcription factor regulates cell division in *Arabidopsis*. Plant Cell. **18:** 3132-3144

Kinkema M, Fan WH, Dong XN (2000) Nuclear localization of NPR1 is required for activation of PR gene expression. Plant Cell **12:** 2339-2350

Kleczkowski K, Schell J (1995) Phytohormone conjugates – nature and function. Crit Rev Plant Sciences **14:** 283-298

Kloek AP, Verbsky ML, Sharma SB, Schoelz JE, Vogel J, Klessig DF, Kunkel BN

(2001) Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (*coi1*) mutation occurs through two distinct mechanisms. Plant J **26:** 509-522

Koornneef A, Pieterse CMJ (2008) Cross talk in defense signaling. Plant Physiol **146:** 839-844

Koorneef M, Alonso-Blanco C, Peeters AJM, Soppe W (1998) Genetic control of flowering time in *Arabidopsis*. Annu. Rev. Plant Physiol Mol Biol **49:** 345-370

Krishna P (2003) Brassinosteroid-mediated stress responses. J Plant Growth Regul **22:** 289-297

Kunkel BN, Bent AF, Dahlbeck D, Innes RW, Staskawicz BJ (1993) RPS2, an Arabidopsis disease resistance locus specifying recognition of Pseudomonas syringae strains expressing the avirulence gene AvrRpt2. Plant Cell **8:** 865-875

Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, Felix G (2004) The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. Plant Cell **16:** 3496-3507

Kus JV, Zaton K, Sarkar R, Cameron RK (2002) Age-related resistance in *Arabidopsis* is a developmentally regulated defense response to *Pseudomonas syringae*. Plant Cell **14**: 479-490

Langlois-Meurinne M, Gachon CMM, Saindrenan P (2005) Pathogen-responsive expression of glycosyltransferase genes *UGT73B3* and *UGT73B5* is necessary for resistance to *Pseudomonas syringae* pv *tomato* in *Arabidopsis*. Plant Physiol **139:** 1890-1901

Laudert D, Weiler EW (1998) Allene oxide synthase: a major control point in Arabidopsis thaliana octadecanoid signaling. Plant J **15:** 675-684

Laufs P, Peacucelle A, Morin H, Traas J (2004) MicroRNA regulation of the CUC genes is required for boundary size control in *Arabidopsis* meristems. Development. **131:** 4311-4322

Lawton KA, Potter SL, Uknes S, Ryals J (1994) Acquired resistance signal transduction in Arabidopsis is ethylene independent. Plant Cell **6:** 581-588

LeClere S, Tellez R, Rampey RA,, Matsuda SPT, Bartel B (2002) Characterization of a family of IAA-amino acid conjugate hydrolases from *Arabidopsis*. J Biol Chem **277:** 20446-20452

Lee I, Aukerman MJ, Gore SL, Lohman KN, Michaels SD, Weaver LM, John MC, Feldmann KA, Amasino RM (1994) Isolation of *LUMINIDEPENDENS*: a gene involved in the control of flowering time in Arabidopsis. Plant Cell **6:** 75-83

Lee JH, Hong SM, Yoo SJ, Park OK, Lee JS, Ahn JH (2006) Integration of floral inductive signals by flowering locus T and suppressor of overexpression of *Constans 1*. Physiologia Plantarum **126:** 475-483

Lee JH, Mitchell RJ, Kim BC, Cullen DC, Gu MB (2005) A cell biosensor for environmental toxicity analysis. Biosensors and Bioelectronics **21:** 500-507

Leisner SM, Turgeon R, Howell SH (1993) Effects of host plant development and genetic determinants on the long-distance movement of cauliflower mosaic virus in *Arabidopsis*. Plant Cell **5:** 191-202

Lemaire SD (2004) The glutaredoxin family in oxygenic photosynthetic organisms.

Photosynthesis Research **79:** 305-318

LeNoble ME, Spollen WG, Sharp RE (2004) Maintenance of shoot growth by endogenous ABA: genetic assessment of the involvement of ethylene suppression. J Exp Bot **55:** 237-245

Leon-Reyes A, Spoel SH, De Lange ES, Abe H, Kobayashi M, Tsuda S, Millenaar FF, Welschen RAM, Ritsema T, Pieterse MJC (2009) Ethylene modulates the role of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 in cross talk between salicylate and jasmonate signaling. Plant Physiol 149: 1797-1809

Levy YY, Dean C (1998) The transition to flowering. Plant Cell **10:** 1973-1989

Lorenzo O, Piqueras R, Sanchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR 1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15:** 165-178

Li D, Liu C, Shen L, Wu Y, Chen H, Robertson M, Helliwell CA, Ito T, Meyerowitz E, Yu H (2008) A repressor complex governs the integration of flowering signals in *Arabidopsis*. Cell **15:** 110-120

Li J, Brader G, Kariola T, Palva ET (2006) WRKY70 modulates the selection of signaling pathways in plant defense. Plant J **46:** 477-491

Li Y, Baldauf S, Lim EK, Bowles DJ (2001) Phylogenetic analysis of the UDP-glycosyltransferase multigene family of *Arabidopsis thaliana*. J Biol Chem **276:** 4338-4343

Lin Z, Zhong S, Grierson D (2009) Recent advances in ethylene research. J Exp Bot **60:** 3311-3336

Liscum E, Reed JW (2002) Genetics of Aux/IAA and ARF action in plant growth and development. Plant Mol Biol **49:** 387-400

Liu X, Yue Y, Li B, Nie Y, Li W, Wu W-H, Ma L (2007) A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. Science **315:** 1712-1716

Ljung K, Hull AK, Kowalczyk M, Marchant A, Celenza J, Cohen JD, Sandberg G (2002) Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. Plant Mol Biol **50:** 309-332

Llorente F, Muskett P, Sanchez-Vallet A, Lopez G, Ramos B, Sanchez-Rodriguez C, Jorda L, Parker J, Molina A (2008) Repression of the auxin response pathway increases *Arabidopsis* susceptibility to necrotrophic fungi. Mol Plant **1:** 496-509

van Loon LC, Geraats BPJ, Linthorst HJM (2006) Ethylene as a modulator of disease resistance in plants. Trends Plant Science **11:** 184-191

Lorenzo O, Chico JM, Sanchez-Serrano JJ, Solano R (2004) *JASMONATE-INSENSITIVE 1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell **16:** 1938-1950

Lorenzo O, Piqueras R, Sanchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15:** 165-178

Lotan T, Ori N, Fluhr R (1989) Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell. **1**: 881-887

Love AJ, Yun BW, Laval V, Loake GJ, Milner JJ (2005) *Cauliflower mosaic virus*, a compatible pathogen of Arabidopsis, engages three distinct defense-signaling pathways and activates rapid systemic generation of reactive oxygen species. Plant Physiol **139:** 935-948

Ma S-W, Morris VL, Cuppels DA (1991) Characterization of a DNA region required for production of the phytotoxin coronatine by *Pseudomonas syringae* pv. *tomato*. MPMI **4:** 69-74

Mackey D, Belkahadir Y, Alonso JM, Ecker JR, Dangl JL (2003) *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. Cell **112:** 379-389

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

Maldonado AM, Doerner P, Dixon RA, Lamb CJ, Cameron RK (2002) A putative lipid transfer protein involved in systemic resistance signaling in Arabidopsis. Nature **419:** 399-403

von Malek B, van der Graaff E, Schneitz K, Keller B (2002) The *Arabidopsis* male-sterile mutant *dde2-2* is defective in the *ALLENE OXIDE SYNTHASE* gene encoding one of the key enzymes of the jasmonic acid biosynthesis pathway. Planta **216:** 187-192

Mallory AC, Dugas DV, Bartel DP, Bartel B (2004) MicroRNA regulation of NAC-

domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. Current Biol. **14:** 1035-1046

Manfield IW, Devlin PF, Jen C-H, Westhead DR, Gilmartin PM (2007) Conservation, convergence, and divergence of light-responsive, circadian-regulated, and tissue-specific expression patterns during evolution of the Arabidopsis GATA gene family. Plant Physiol **143:** 941-958

McCormack E, Tsai Y-C, Braam J (2005) Handling calcium signaling: *Arabidopsis* CaMs and CMLs. Trends Plant Science **10:** 383-389

McDowell JM, Williams SG, Funderburg NT, Eulgem T, Dangl JL (2005) Genetic analysis of developmentally regulated resistance to downy mildew (*Hyaloperonospora parasitica*) in *Arabidopsis thaliana*. MPMI **18:** 1226-1234

McGrath KC, Dombrecht B, Manners JM, Schenk PM, Edgar CI, Maclean DJ, Scheible W-R, Udvardi MK, Kasan K (2005) Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of *Arabidopsis* transcription factor gene expression. Plant Physiol **139:** 949-959.

Melotto M, Mecey C, Niu Y, Chung HS, Katsir L, Yao J, Zeng W, Thines B, Staswick P, Browse J, Howe GA, He SY (2008) A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. Plant J **55:** 979-988

Melotto M, Underwood W, Koczan J, Nomura K, He SY (2006) Plant stomata

function in innate immunity against bacterial invasion. Cell **126:** 969-980

Menke FLH, van Pelt JA, Pieterse CMJ, Klessig DF (2004) Silencing of the mitogen-activated protein kinase MPK6 compromises disease resistance in Arabidopsis. Plant Cell **16:** 897-907

Metraux JP, Signer H, Ryals J, Ward E, Wyssbenz M, Gaudin J, Raschdorf K, Schmid E, Blum W, Inverardi B (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science **250:** 1004-1006

Michaels SD, Amasino RM (1999) *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell. **11:** 949-956

Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2005) The NAC transcription factors NST1 and NST2 of *Arabidopsis* regulate secondary wall thickenings and are required for anther dehiscence. Plant Cell **17:** 2993-3006

Mohr PG, Cahill DM (2007) Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in *Arabidopsis* infected with *Pseudomonas syringae* pv. *tomato*. Funct Integr Genomics **7:** 181-191

Montesinos E, Moragrega C, Llorente I, Vilardell P (1995) Susceptibility of selected European pear cultivars to infection by *Stemphylium vesicarium* and influence of leaf and fruit age. Plant Disease **79**: 471-473

Mou Z, Fan WH, Dong XN (2003) Inducers of plants systemic acquired resistance regulate NPR1 function through redox changes. Cell **113:** 935-944

Mueller S, Hilbert B, Dueckershoff K, Roitsch T, Krischke M, Mueller MJ, Berger

S (2008) General detoxification and stress responses are mediated by oxidized lipids through TGA transcription factors in *Arabidopsis*. Plant Cell **20:** 768-785

Mysore KS, Crasta OR, Tuori RP, Folkerts O, Swirsky PB, Martin GB (2002) Comprehensive transcript profiling of Pto- and Prf-mediated host defense responses to infection by *Pseudomonas syringae* pv. *tomato*. Plant J **32:** 299-315

Nafisi M, Goregaoker S, Botanga CJ, Glawischnig E, Olsen CE, Halkier BA, Glazebrook J (2007) *Arabidopsis* Cytochrome P450 Monooxygenase 71A13 Catalyzes the Conversion of Indole-3-Acetaldoxime in Camalexin Synthesis. Plant Cell **19:** 2039–2052

Nagpal P, Ellis CM, Weber H, Ploense SE, Barkawi LS, Guilfoyle TJ, Hagen G, Alonso JM, Cohen JD, Farmer EE, Ecker JR, Reed JW (2005) Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development **132:** 4107-4118

Nakashita H, Yasuda M, Nitta T, Asami T, Fujoka S, Arai Y, Sekimata K, Takasuto S, Yamaguchi I, Yoshida S (2003) Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. Plant J **33:** 887-898

Navarro L, Zipfel C, Rowland O, Keller I, Robatzek S, Boller T, Jones JDG (2004) The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. Plant Phys **135:** 1113-1128

Nawrath C, Heck S, Parinthawong N, Metraux JP (2002) EDS5, an essential

component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. Plant Cell **14:** 275–286

Nawrath C, Metraux J-P (1999) Salicylic Acid Induction-Deficient Mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. Plant cell **11:** 1393-1404

Ndamukong I, Al Abdallat A, Thurow C, Fode B, Zander M, Weigel R, Gatz C (2007) SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive *PDF1.2* transcription. Plant J **50:** 128-139

Negi S, Ivanchenko MG, Muday GK (2008) Ethylene regulates lateral root formation and auxin transport in *Arabidopsis thaliana*. Plant J **55:** 175-187

Nicaise V, Roux M, Zipfel C (2009) Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. Plant Phys **150:** 1638-1647

Nickstadt A, Thomma BPHJ, Feussner I, Kangasjarvi J, Zeier J, Loeffler C, Scheel D, Berger S (2004) The jasmonate-insensitive mutant *jin1* shows increased resistance to biotrophic as well as necrotrophic pathogens. Mol Plant Pathol **5:** 425-434

Nikovics K, Blein T, Peaucelle A, Ishida T, Morin H, Aida M, Laufs P (2006) The balance between the *MIR16A* and *CUC2* genes controls leaf margin serration in *Arabidopsis*. Plant Cell **18:** 2929-2945

Nomura K, Melotto M, He S-Y (2005) Suppression of host defense in compatible plant-

Pseudomonas syringae interactions. Curr Opin Plant Biol **8:** 361-368

Nomura T, Kushiro T, Yokota T, Kamiya Y, Bishop GJ, Yamaguchi S (2005) The last reaction producing brassinolide is catalyzed by cytochrome P-450s, CYP85A3 in tomato and CYP85A2 in *Arabidopsis*. J Biol Chem **280:** 17873-17879

Oh S-K, Lee S, Yu SH, Choi D (2005) Expression of a novel NAC domain-containing Transcription factor (CaNAC1) is preferentially associated with incompatible interactions between chili pepper and pathogens. Planta **222:** 876-887

Ohnishi T, Sugahara S, Yamada T, Kikuchi K, Yoshiba Y, Hirano H-Y, Tsutsumi N (2005) *OsNAC6*, a member of the *NAC* gene family, is induced by various stresses in rice. Genes Genet Syst **80:** 135-139

Olsen AN, Ernst HA, Leggio LL, Skriver K (2005) NAC transcription factors: structurally distinct, functionally diverse. Trends Plant Sci **10**: 79-87

Ooka H, Satoh K, Doi K, Nagata T, Otomo Y, Murakami K, Matsubara K, Osato N, Kawai J, Carninci P, Hayashizaki Y, Suzuki K, Kojima K, Takahara Y, Yamamoto K, Kikuchi S (2003) Comprehensive analysis of *NAC* family genes in *Oryza sativa* and *Arabidopsis thaliana*. DNA Research **10:** 239-247

Pandey S, Nelson DC, Assmann SM (2009) Two novel GPCR-type G proteins are abscisic acid receptors in *Arabidopsis*. Cell **136:** 136-148

Pandey SP, Somssich IE (2009) The role of WRKY transcription factors in plant immunity. Plant Phys **150:** 1648-1655

Panter SN, Hammond-Kosack KE, Harrison K, Jones JDG, Jones DA (2002)

Developmental control of promoter activity is not responsible for mature onset of *Cf-9B*-mediated resistance to leaf mold in tomato. MPMI **15:** 1099-1107

Panter SN, Jones DA (2002) Age-related resistance to plant pathogens. Advances in Botanical Research **38:** 251-280

Park J-H, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. Plant J **31:** 1-12

Park JM, Park C-J, Lee S-B, Ham B-K, Shin R, Paek K-H (2001) Overexpression of the tobacco *Tsi1* gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. Plant Cell **13:** 1035-1046

Park S-W, Kaimoyo E, Kumar D, Mosher S, Klessig DF (2007) Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science **318:** 113-116

Park S-Y, Fung P, Nishimura N, Jensen DR, Fuji H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow T-FF, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rodriguez PL, McCourt P, Zhu J-K, Schroeder JI, Volkman BF, Cutler SR (2009) Abscisic acid inhibits type 2C protein Phosphatase via the PYR/PYL family of START proteins. Science **324:** 1068-1071

Penninckx IAMA, Eggermont K, Terras FRG, Thomma BPHJ, De Samblanx GW,

Buchala A, Metraux J-P, Manners JM, Broekaert WF (1996) Pathogen-induced systemic activation of a plant Defensin gene in Arabidopsis follows a salicylic acid-independent pathway. Plant Cell **8:** 2309-2323

Penninckx IAMA, Thomma BPHJ, Buchala A, Metraux J-P, Broekaert WF (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell **10:** 2103-2113

Petersen BL, Chen S, Hansen CH, Olsen CE, Halkier BA (2002) Composition and content of glucosinolates in developing *Arabidopsis thaliana*. Planta **214**: 562-571

Petnicki-Ocwieja T, Schneider DJ, Tam VC, Chancey ST, Shan L, Jamir Y, Schechter LM, Janes MD, Buell CR, Tang X, Collmer A, Alfano JR (2002) Genomewide identification of proteins secreted by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. *tomato* DC3000. PNAS **99:** 7652-7657

Pieterse CMJ, Van Loon LC (2004) NPR1: the spider in the web of induced resistance signaling pathways. Curr Opin Plant Biol **7:** 456-464

Pitzschke A, Schikora A, Hirt H (2009) MAPK cascade signaling networks in plant defence. Current Opin Plant Biol **12:** 421-426

Porta H, Rocha-Sosa M (2002) Plant lipoxygenases. Physiological and molecular features. Plant Physiol **130:** 15-21

Prithiviraj B, Bais HP, Jha AK, Vivanco JM (2005) *Staphylococcus aureus*

pathogenicity on *Arabidopsis thaliana* is mediated either by a direct effect of salicylic acid on the pathogen or by SA-dependent, NPR1-independent host responses. Plant J **42:** 417-432

Putterill J, Robson F, Lee K, Simon R, Coupland G (1995) The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc-finger transcription factors. Cell. **80:** 847-857

Qiao H, Chang KN, Yazaki J, Ecker JR (2009) Interplay between ethylene, ETP1/ETP2 F-box proteins, and degradation of EIN2 triggers ethylene responses in *Arabidopsis*. Genes Dev **23:** 512-521

Qiu J-L, Fiil BK, Petersen K, Nielsen HB, Botanga CJ, Thorgrimsen S, Palma K, Suarez-Rodriguez MC, Sandbech-Clausen S, Lichota J, Brodersen P, Grasser KD, Mattsson O, Glazebrook J, Mundy J, Petersen M (2008) *Arabidopsis* MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. EMBO **27:** 2214-2221

Quirino BF, Bent AF (2003) Deciphering host resistance and pathogen virulence: the *Arabidopsis/Pseudomonas* interaction as a model. MPP **4:** 517-530

Quirino BF, Normanly J, Amasino RM (1999) Diverse range of gene activity during *Arabidopsis thaliana* leaf senescence includes pathogen-independent induction of defense-related genes. Plant Mol Biol **40**: 267-278

Raacke IC, Mueller MJ, Berger S (2006) Defects in allene oxide synthase and 12-oxa-photodienoic acid reductase alter the resistance to *Pseudomonas syringae* and *Botrytis cinerea*. J Phytopath **154:** 740-744

Rahman A, Hosokawa S, Oono Y, Amakawa T, Goto N, Tsurumi S (2008) Auxin and ethylene response interactions during Arabidopsis root hair development dissected by auxin influx modulators. Plant Physiol **130:** 1908-1917

Raffaele S, Rivas S, Roby D (2006) An essential role for salicylic acid in AtMYB30-mediated control of the hypersensitive cell death program in Arabidopsis. FEBS Letters **580:** 3498-3504

Razem FA, El-Kereamy A, Abrams SR, Hill RD (2006) The RNA-binding protein FCA is an abscisic acid receptor. Nature **439:** 290-294

Redei GP (1962) Supervital mutants in Arabidopsis. Genetics **47:** 443-460

Ren C, Pan J, Pen W, Genschik P, Hobbie L, Hellmann H, Estelle M, Gao B, Peng J, Sun C, Xie D (2005) Point mutations in Arabidopsis *Cullin1* reveals its essential role in jasmonate response. Plant J **42:** 514-524

Ren D, Yang K-Y, Li G-J, Liu Y, Zhang S (2006) Activation of Ntf4, a tobacco mitogen-activated protein kinase, during plant defense response and its involvement in hypersensitive response-like cell death. Plant Physiol **141:** 1482-1493

Ren T, Qu F, Morris TJ (2000) *HRT* gene function requires interaction between a NAC protein and viral capsid protein to confer resistance to turnip crinkle virus. Plant Cell **12:** 1917-1925

Rochon A, Boyle P, Wignes T, Fobert PR, Despres C (2006) The coactivator function of *Arabidopsis* NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. Plant Cell **18:** 3670-3685

Roumen EC, Bonman JM, Parlevliet JE (1992) Leaf age related partial resistance to *Pyricularia oryzae* in tropical lowland rice cultivars as measured by the number of sporulating lesions. Phytopathology **82**:1414-1417.

Ruiz-Medrano R, Xoconoste-Cazares B, Lucas WJ (1999) Ploem long-distance transport of *CmNACP* mRNA: Implications for supracellular regulation in plants. Development **126:** 4405-4419

Rushton PJ, Somssich IE (1998) Transcriptional control of plant genes responsive to pathogens. Curr Opin Plant Biol **1:** 311-315

Rusterucci C, Zhao A, Haines K, Mellersh D, Neumann M, Cameron RK (2005) Age-related resistance to *Pseudomonas syringae pv. Tomato* is associated with the transition to flowering in *Arabidopsis* and is effective against *Peronospora parasitica.* PMPP **66**: 222-231

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner H-Y, Hunt MD (1996) Systemic acquired resistance. Plant Cell **8:** 1809-1819

Sablowski RWM, Meyerowitz EM (1998) A homolog of *NO APICAL MEISTEM* is an immediate target of the floral homeotic genes *APETALA3/PISTILLATA*. Cell 92: 93-103

Salzman RA, Tikhonova I, Bordelon BP, Hasegawa PM, Bressan RA (1998) Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense response during fruit ripening in grape. Plant Physiol **117**: 465-472

Sanders PM, Lee PY, Biesgen C, Boone JD, Beals TP, Weiler EW, Goldberg RB

(2000) The Arabidopsis *DELAYED DEHISCENCE1* gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell **12:** 1041-1061

Sasaki-Sekimoto Y, Taki N, Obayashi T, Aono M, Matsumoto F, Sakurai N, Suzuki H, Hirai MY, Noji M, Saito K, Masuda T, Takamiya K-I, Shibata D, Ohta H (2005) Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. Plant J **44:** 653-668

Schechter LM, Vencato M, Jordan KL, Schneider SE, Schneider DJ, Collmer A (2006) Multiple approaches to a complete inventory of *Pseudomonas syringae* pv. *tomato* DC3000 type III secretion system effector proteins. MPMI **19:** 1180-1192

Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, Manners JM (2000) Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. PNAS **97:** 11655-11660

Schmelz EA, Engelberth J, Alborn HT, O'Donnell P, Sammons M, Toshima H, Tumlinson III JH (2003) Simultaneous analysis of phytohormones, phytotoxins, and volatile organic compounds in plants. PNAS **100:** 10552-10557

Schmitz RJ, Sung S, Amasino RM (2008) Histone arginine methylation is required for vernalization-induced epigenetic silencing of *FLC* in winter-annual *Arabidopsis thaliana*. PNAS **105:** 411-416

Schuhegger R, Ihring A, Ganter S, Bahnweg G, Knappe C, Vogg G, Hutzler P,

Schmid M, Van Breusegem F, Eberl L, Hartmann A, Langebartels C (2009) Induction of systemic resistance in tomato by N-acyl-L-homoserine lactone-producing rhizosphere bacteria. Plant Cell and Environment **29:** 909-918

Schulaev V, Silverman P, Raskin I (1997) Airborne signaling by methyl salicylate in plant pathogen resistance. Nature **385:** 718-721

Schuler MA, Werck-Reichhart D (2003) Functional genomics of P450s. Annu Rev Plant Biol **54:** 629-667

Selth LA, Dogra SC, Rasheed MS, Healy H, Randles JW, Rezaian MA (2005) A NAC domain protein interacts with *Tomato Leaf Curl Virus* replication accessor protein and enhances viral replication. Plant Cell **17:** 311-325

Shah J, Tsui F, Klessig DF (1997) Characterization of a salicylic acid-insensitive mutant (*sai*) of *Arabidopsis thaliana* identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. MPMI **10:** 69-78

Shaik M, Dickinson TA, Steadman JR (1989a) Variation in rust susceptibility in beans: predicting lesion size from leaf developmental stage measured by leaf age, length, and plastochron index. Phytopathology **79**: 1035-1042

Shaik M, Steadman JR (1989b) The effect of leaf developmental stage on the variation of resistant and susceptible reactions of *Phaseolus vulgaris* to *Uromyces appendiculatus*. Phytopathology **79**: 1028-1035.

Shearer HL, Wang L, DeLong C, Despres C, Fobert PR (2009) NPR1 enhances the DNA binding activity of the *Arabidopsis* bZIP transcription factor TGA7. Botany **87:** 561-570

Sheen J (1998) Mutational analysis of protein phosphatase 2C involved in abscisic acid signal transduction in higher plants. PNAS **95:** 975-980

Sheldon CC, Rouse DT, Finnegan J, Peacock WJ, Dennis ES (2000) The molecular basis of vernalization: the central role of *FLOWERING LOCUS C* (*FLC*). PNAS **97:** 3753-3758

Shen Y-Y, Wang X-F, Wu F-Q, Du S-Y, Cao Z, Shang Y, Wang X-L, Peng C-C, Yu X-C, Zhu S-Y, Fan R-C, Xu Y-H, Zhang D-P (2006) The Mg-chelatase H subunit is an abscisic acid receptor. Nature **443:** 823-826

Sikkema J, Bont JAM, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiology Reviews **59:** 201-222

Simpson GG, Dean C (2002) *Arabidopsis*, the rosetta stone of flowering time? Science **296:** 285-289

Sirjusingh C, Sutton JC, and Tsujita MJ (1996) Effects of inoculum concentration and host age on infection of geranium by *Botrytis cinerea.* Plant Disease **80**:154-159

Slaymaker DH, Navarre DA, Clark D, del Pozo O, Martin GB, Klessig DF (2002) The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant activity and plays a role in the hypersensitive defense response. PNAS **99:** 11640-11645

Smyczynski C, Roudier F, Gissot L, Vaillant E, Grandjean O, Morin H, Masson T, Bellec Y, Geelen D, Faure J-D (2006) The c terminus of the immunophilin PASTICCINO1 is required for plant development and for interaction with a NAC-like transcription factor. J Biol Chemistry **281:** 25475-25484

Solano R, Stepanova A, Chao Q, Ecker JR (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev **12:** 3703-3714

Song JT, Koo YJ, Seo HS, Kim MC, Choi YD, Kim JH (2008) Overepxression of AtSGT1, an Arabidopsis salicylic acid glucosyltransferase, leads to increased susceptibility to *Pseudomonas syringae*. Phytochem **69:** 1128-1134

Souer E, van Houwelingen A, Kloos D, Mol J, Koes R (1996) The *No Apical Meristem* gene of petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordial boundaries. Cell **85:** 159-170

Spoel SH, Johnson JS, Dong X (2007) Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. PNAS **104:** 18842-18847

Spoel SH, Koornneef A, Claessens SMC, Korzelius JP, van Pelt JA, Mueller MJ, Buchala AJ, Metraux J-P, Brown R, Kazan K, van Loon LC, Dong X, Pieterse CMJ (2003) NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15:** 760-770

Staswick PE, Serban B, Rowe M, Tiryaki I, Maldonado MT, Maldonado MC, Suza W (2005) Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. Plant Cell **17:** 616-627

Staswick PE, Tiryaki I (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell **16:** 2117-2127

Staswick PE, Tiryaki I, Rowe ML (2002) Jasmonate response locus *JAR1* and several

related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell. **14:** 1405-1415

Shtienberg D, Kremer Y, Dinoor A (1993) Influence of physiological age of pima cotton on the need for fungicide treatment to suppress atlernaria leaf spot. Phytopathol **83**: 1235-1239

Stintzi A, Browse J (2000) The *Arabidopsis* male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. PNAS **97:** 10625-10630

Stintzi A, Weber H, Reymond P, Browse J, Farmer EE (2001) Plant defense in the absence of jasmonic acid: the role of cyclopentenones. PNAS **98:** 12837-12842

Stenzel I, Hause B, Miersch O, Kurz T, Maucher H, Weichert H, Ziegler J, Feussner I, Wasternack C (2003) Jasmonate biosynthesis and the allene oxide cyclase family of *Arabidopsis thaliana*. Plant Mol Biol **51:** 895-911

Stepnova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie D-Y, Dolezal K, Schlereth A, Jurgens G, Alonso JM (2008) *TAA1*-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell **133:** 177-191

Suarez-Rodriguez MC, Adams-Phillips L, Liu Y, Wang h, Su S-H, Jester PJ, Zhang S, Bent AF, Krysan PJ (2007) MEKK1 is required for flg22-induced MPK4 activation in Arabidopsis plants. Plant Phys **143:** 661-669

Takada S, Hibara K, Ishida T, Tasaka M (2001) The *CUP-SHAPED COTYLEDON1*

gene of *Arabidopsis* regulates shoot apical meristem formation. Development **128:** 1127-1135

Takahashi F, Yoshida R, Ichimura K, Mizoguchi T, Seo S, Yonezawa M, Maruyama K, Yamaguchi-Shinozaki K, Shinozaki K (2007) The mitogen-activated protein kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction pathway in *Arabidopsis*. Plant Cell **19:** 805-818

Tam YY, Epstein E, Normanly J (2000) Characterization of auxin conjugates in Arabidopsis. Low steady-state levels of indole-3-acetyl-aspartate, indole-3-acetyl-glutamate, and indole-3-acetyl-glucose. Plant Physiol **123:** 589-595

Tao Z, Liu H, Qiu D, Zhou Y, Li X, Xu C, Wang S (2009) A pair of allelic WRKY genes play opposite roles in rice-bacteria interactions. Plant Phyiol **151:** 936-948

Taoka K, Yanagimoto Y, Daimon Y, Hibara K, Aida M, Tasaka M (2004) The NAC domain mediates functional specificity of CUP-SHAPED COTYLEDON proteins. Plant J **40:** 462-473

Teakle GR, Manfield IW, Graham JF, Gilmartin PM (2002) *Arabidopsis thaliana* GATA factors: organization, expression and DNA-binding characteristics. Plant Mol Biol **50:** 43-47

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signaling. Nature **448:** 661-665

Thomma BPHJ, Eggermont K, Tierrens FM-J, Broekaert WF (1999) Requirement of

functional *Ethylene-Insensitive 2* gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. Plant Physiol **121:** 1093-1101

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nuc Acid Res **22:** 4673

Thurow C, Schiermeyer A, Krawczyk S, Butterbrodt T, Nickolov K, Gatz C (2005) Tobacco bZIP transcription factor TGA2.2 and related factor TGA2.1 have distinct roles in plant defense responses and plant development. Plant J **44:** 100-113

Tiryaki I, Stswick PE (2002) An Arabidopsis mutant defective in jasmonate response is allelic to the auxin-signaling mutant *axr1*. Plant Physiol **130:** 887-894

Ton J, Flors V, Mauch-Mani B (2009) The multifaceted role of ABA in disease resistance. Trends Plant Science **14:** 310-317

Ton J, Mauch-Mani B (2004) β-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. Plant J **38:** 119-130

Toppan A, Roby D, Esquerre-Tugaye M-T (1982) Cell surfaces in plant-microorganism interactions. Plant Phys **70:** 82-86

Tor M, Brown D, Cooper A, Wooks-Tor, Sjolander K, Jones JDG, Holub EB (2004) Arabidopsis downy mildew resistance gene *RPP27* encodes a receptor-like protein similar to *CLAVATA2* and tomato *Cf-9*. Plant Phys **135:** 1100-1112

de Torres Zabala M, Bennett MH, Truman WH, Grant MR (2009) Antagonism

between salicylic and abscisic acid reflects early host-pathogen conflict and moulds plant defence responses. Plant J **59:** 375-386

de Torres-Zabala M, Truman W, Bennett MH, Lafforgue G, Mansfield JW, Egea PR, Bogre L, Grant M (2007) *Pseudomonas syringae* pv. *tomato* hijacks the *Arabidopsis* abscisic acid signaling pathway to cause disease. EMBO. **26:** 1434-1443

Tran LSP, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the *early responsive to dehydration stress 1* promoter. Plant Cell **16:** 2481-2498

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

Tsuda k, Sato M, Galzebrook J, Cohen JD, Katagiri F (2008) Interplay between MAMP-triggered and SA-mediated defense responses. Plant J **53:** 763-775

Turner JG, Ellis C, Devoto A (2002) The jasmonate signal pathway. Plant Cell S153-S164

Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. PNAS **98:** 5116-5121

Uknes S, Winter A, Delaney T, Vernooij B, Morse A, Friedrich L (1993) Biological induction of systemic acquired resistance in *Arabidopsis*. MPMI **6**: 692-8

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J. (2006) A NAC gene regulating senescence improves grain protein, zinc and iron content in wheat. Science **314:** 1298-1301

Van Bel AJE, Gaupels F (2004) Pathogen-induced resistance and alarm signals in the phloem. MPP **5:** 495-504

VanderMolen GE, Labavitch JM, Strand LL, DeVay JE (1983) Pathogen-induced vascular gels: ethylene as a host intermediate. Physiol Plant **59:** 573-580

Vernooij B, Friedrich L, Morse A, Reist R, Kolditzjawhar R, Ward E, Uknes S, Kessmann H, Ryals J (1995) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell **6:** 959-65

Vincenzetti S, Cambi A, Neuhard J, Schnorr K, Grelloni M, Vita A (1999) Cloning, expression, and purification of cytidine deaminase from *Arabidopsis thaliana.* Protein Expression and Purification **15:** 8-15

Vlot AC, Dempsey DA, Klessig DF (2009) Salicylic acid, a multifaceted hormone to combat disease. Annu Rev Phytopath **47:** 177-206

Vroemen CW, Mordhorst AP, Albrecht C, Kwaaitaal MACJ, de Vries SC (2003) The *CUP-SHAPED COTYLEDON3* gene is required for boundary and shoot meristem formation in *Arabidopsis*. Plant Cell **15:** 1563-1577

Wang D, Pajerowska-Mukhtar Karolina, Culler AH, Dong X (2007) Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. Current Biology. **17:** 1784-1790

Wang L, Mitra RM, Hasselmann KD, Sato M, Lenarz-Wyatt L, Cohen JD, Katagiri F, Glazebrook (2008) The genetic network controlling the *Arabidopsis* transcriptional response to *Pseudomonas syringae* pv. *maculicola*: roles of major regulators and the phytotoxin coronatine. MPMI **21:** 1408-1420

Wang L, Silke A, Wu J, Baldwin IT (2008) Comparison of LIPOXYGENASE3- and JASMONATE-RESISTANT4/6-silenced plants reveal that jasmonic acid and jasmonic acid-amino acid conjugates play different roles in herbivore resistance of *Nicotiana attenuata*. Plant Physiol **146:** 904-915

Wang L, Tsuda K, Sato M, Cohen JD, Katagiri F, Glazebrook J (2009) Arabidopsis CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against *Pseudomonas syringae*. PLoS Pathogen **5:** e1000301

Wang X, Basnayake BMVS, Zhang H, Li G, Li W, Virk N, Mengiste T, Song F (2009) The *Arabidopsis* ATAF1, a NAC transcription factor, is a negative regulator of defense responses against necrotrophic fungal and bacterial pathogens. MPMI **22:** 1227-1238

Ward EWB, Cahill DM, Bhattacharyya MK (1989) Abscisic acid suppression of phenylalanine ammonia-lyase activity and mRNA, and resistance of soybeans to *Phytophthora megasperma* f.sp. *glycinea*. Plant Physiol **91:** 23-27

Wasilewska A, Vlad F, Sirichandra C, Redko Y, Jammes F, Valon C, dit Frey NF, Leung J (2008) An update on abscisic acid signaling in plants and more. Mol Plant **1:** 198-217

Weigel RR, Pfitzner UM, Gatz C (2005) Interaction of NIMIN1 with NPR1 modulates *PR* gene expression in Arabidopsis. Plant Cell **17:** 1279-1291

Weingart H, Ullrich H, Geider K, Volksch B (2001) The role of ethylene production in virulence of *Pseudomonas syringae* pvs. *glycinea* and *phaseolicola.* Phytopathology. **91:** 511-518

Whalen MC (2005) Host defence in a developmental context. MPP **6:** 347-360

Whalen MC, Innes RW, Bent AF, Staskawicz BJ (1991) Identification of Pseudomonas syringae pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. Plant Cell **3:** 49-59.

Whenham RJ, Fraser RSS, Brown LP, Payne JA (1986) Tobacco-mosaic-virus-induced increase in abscisic-acid concentration in tobacco leaves. Planta **168:** 592-598

Wiermer M, Feys BJ, Parker J (2005) Plant immunity: the EDS1 regulatory node. Curr Opin Plant Biol **8:** 383-389

Wildermuth MC (2006) Variations on a theme: synthesis and modification of plant benzoic acids. Curr Opinion Plant Biol **9:** 288-296

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defense. Nature **414:** 562-5

Wolfe J, Hutcheon C, Higgins V, Cameron RK (2000) A functional gene-for-gene instruction is required for the production of an oxidative burst in response to infection with avirulent *Pseudomonas syringae* pv. *tomato* in *Arabidopsis thaliana.* PMPP **56:** 253-261

Woo H-H, Jeong BR, Hirsch AM, Hawes MC (2007) Characterization of *Arabidopsis* AtUGT85A and AtGUS gene families and their expression in rapidly dividing tissues. Genomics **90:** 143-153

Woodward AW, Bartel B (2005) Auxin: regulation, action, and interaction. Annals Botany **95:** 707-735

Wyatt SE, Pan SQ, Ku J (1991) ß-1,3-glucanase, chitinase, and peroxidase activities in tobacco tissues resistant and susceptible to blue mould as related to flowering, age, and sucker development. PMPP **39:** 433-440

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

Xie Q, Frugis G, Colgan D, Chua N (2000) *Arabidopsis* NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. Genes and Development **14:** 3024-3036

Xu J, Li Y, Wang Y, Liu H, Yang H, Liu G, Ren D (2008) Activation of MAPK kinase 9 induces ethylene and camalexin biosynthesis and enhances sensitivity to salt stress in *Arabidopsis*. J Biol Chem **283:** 26996-27006

Xue G-P, Bower NI, McIntyre CL, Riding GA, Kazan K, Shorter R (2006) TaNAC69 from the NAC superfamily of transcription factors is up-regulated by abiotic stresses in wheat and recognizes two consensus DNA-binding sequences. Functional Plant Biol **33:** 43-57

Yalpani N, Altier DJ, Barbour E, Cigan AL, Scelonge CJ (2001) Production of 6-

methylsalicylic acid by expression of a fungal polyketide synthase activates disease resistance in tobacco. Plant Cell **13:** 1401-1409

Yalpani N, Schulz M, Davis MP, Balke NE (1992) Partial purification and properties of an inducible uridine 5'-diphosphate-glucose:salicylic acid glucosyltransferase from oat roots. Plant Physiol. **100:** 457-463

Yalpani N, Shulaev V, Raskin I (1993) Endogenous salicylic acid levels correlate with accumulation of pathogenesis-related proteins and virus resistance in tobacco. Phytopathol **83**, 702-708

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

Yan J, Zhang C, Gu M, Bai Z, Zhang W, Qi T, Cheng Z, Peng W, Luo H, Nan F, Wang Z, Xie D (2009) The Arabidopsis CORONTINE INSENSITIVE1 protein is a jasmonate receptor. Plant Cell. **21:** 2220-2236

Yan Y, Stolz S, Chetelat A, Reymond P, Pagni M, Dubugnon, Farmer EE (2007) A downstream mediator in the growth repression limb of the jasmonate pathway. Plant Cell **19:** 2470-2483

Yasuda M, Ishikawa A, Jikumaru Y, Seki M, Umezawa T, Asami T, Maruyama-Nakashita A, Kudo T, Shinozaki K, Yoshida S, Nakashita H (2008) Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in *Arabidopsis*. Plant Cell **20:** 1678-1692

Yu GL, Katagiri F, Ausubel FM (1993) Arabidopsis mutations at the RPS2 locus

result in loss of resistance to *Pseudomonas syringae* strains expressing the avirulence gene AvrRpt2. MPMI **6:** 434-443

Yuan J, He SY (1996) The *Pseudomonas syringae* Hrp regulation and secretion system controls the production and secretion of multiple extracellular proteins. J Bacterial **178:** 6399-6402

Zhang J, Shao F, Li Y, Cui H, Chen L, Li H, Zou Y, Long C, Lan L, Chai J, Chen S, Tang X, Zhou J-M (2007) A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. Cell Host Microbe **1:** 175-185

Zhang X, Dai Y, Xiong Y, DeFraia C, Li J, Dong X, Mou Z (2007) Overexpression of Arabidopsis *MAP kinase kinase 7* leads to activation of plant basal and systemic acquired resistance. Plant J **52:** 1066-1079

Zhang Y, Cheng YT, Qu N, Zhao Q, Bi D, Li X (2006) Negative regulation of defense responses in *Arabidopsis* by two *NPR1* paralogs. Plant J **48:** 647-656

Zhang Y, Fan W, Kinkema M, Li X, Dong X (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. PNAS **96:** 6523-6528

Zhao Y, Thilmony R, Bender CL, Schaller A, He SY, Howe GA (2003) Virulence systems of *Pseudomonas syringae* pv. *tomato* promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. Plant J **36:** 485-499

Zhong R, Demura T, Ye ZH (2006) SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of *Arabidopsis*. Plant Cell **18:** 3158-3170

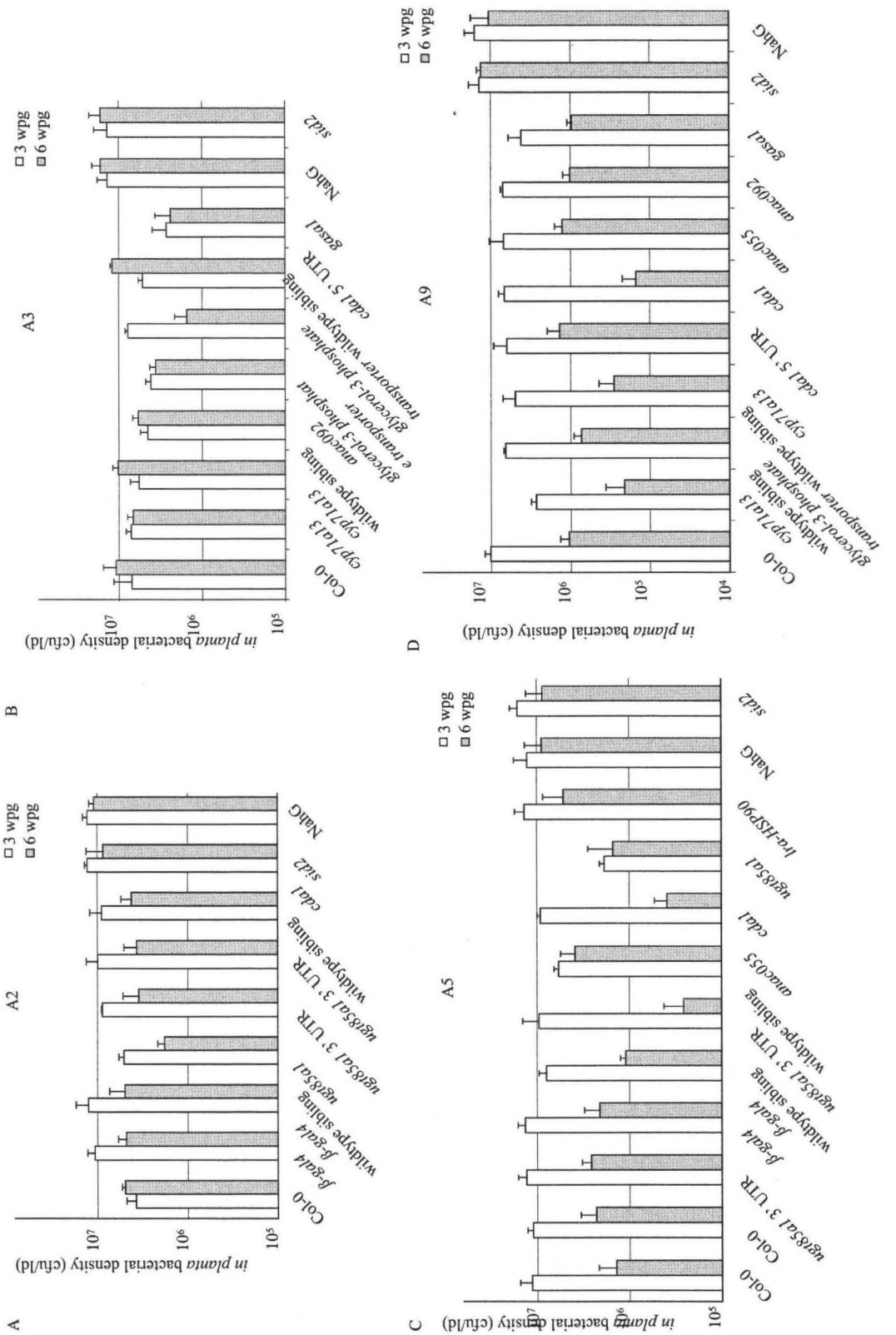
Zhou N, Tootle TL, Tsui F, Klessig DF, Glazebrook J (1998) *PAD4* functions upstream from salicylic acid to control defense responses in Arabidopsis. Plant Cell **10:** 1021-1030

Zimmermann R, Werr W (2005) Pattern formation in the monocot embryo as revealed by *NAM* and *CUC3* orthologues from *Zea mays* L. Plant Mol Biol **58:** 669-685

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T, Felix G (2006) Perception of the bacterial PAMP EF-TU by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell **125:** 749-760

Appendix A: Experiments

Fig A1: *In planta Pst* growth (logarithmic scale) in young (3 wpg) and mature (6 wpg) T-DNA insertion mutant plants of some microarray genes (*β-gal4*, *ugt85a1*, *cda1*, *cyp71a13*, *glycerol-3-transporter*, *gasa1*, *anac055*, *anac092*), *lra* (*HSP90*), wild-type, *sid2*, and *NahG* plants. ARR experiments A) A2, B) A3, C) A5, D) A9, and E) A12 are shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.


A
B
C
D
A2
A3
A5
A9
□ 3 wpg
■ 6 wpg
in planta bacterial density (cfu/ld)
Col-0
pβ-gal4
wildtype sibling
ugt85a1
ugt85a1 3' UTR
cdu1
sid2
NahG
cyp71a13
ume092
glycerol-3 phosphate transporter
cdu1 5' UTR
gasa1
ume055
ugt85a1
Ina-HSP90

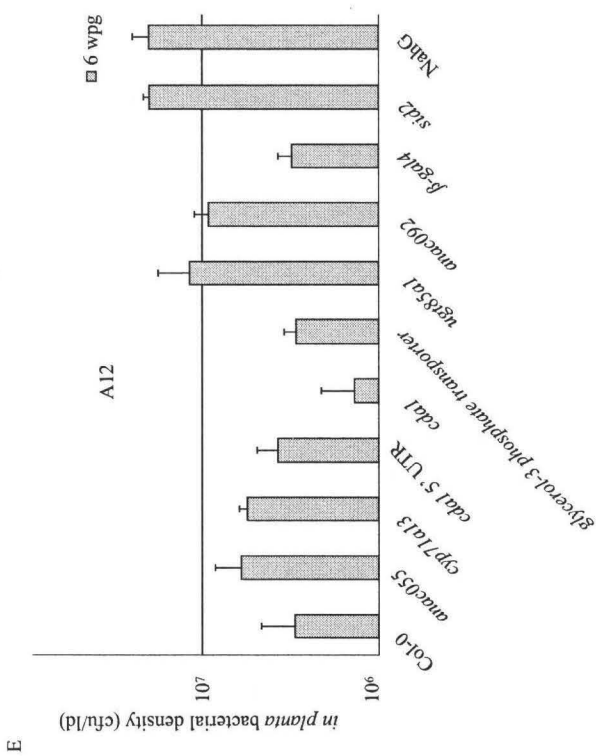

E
in planta bacterial density (cfu/ld)
10^6
10^7
A12
6 wpg
Col-0
amac05
cyp71a13
cda1 5' UTR
cda1
glycerol-3 phosphate transporter
ugt85a1
amac092
β-gal4
sid2
NahG

Fig A2: *In planta Pst* growth (logarithmic scale) in Col-0, *ugt85a1*, *cyp71a13*, *cda1*, and *sid2* at 3, 5, and 6 wpg. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1. ARR experiments A) A27, B) A29, and C) A42 are shown.

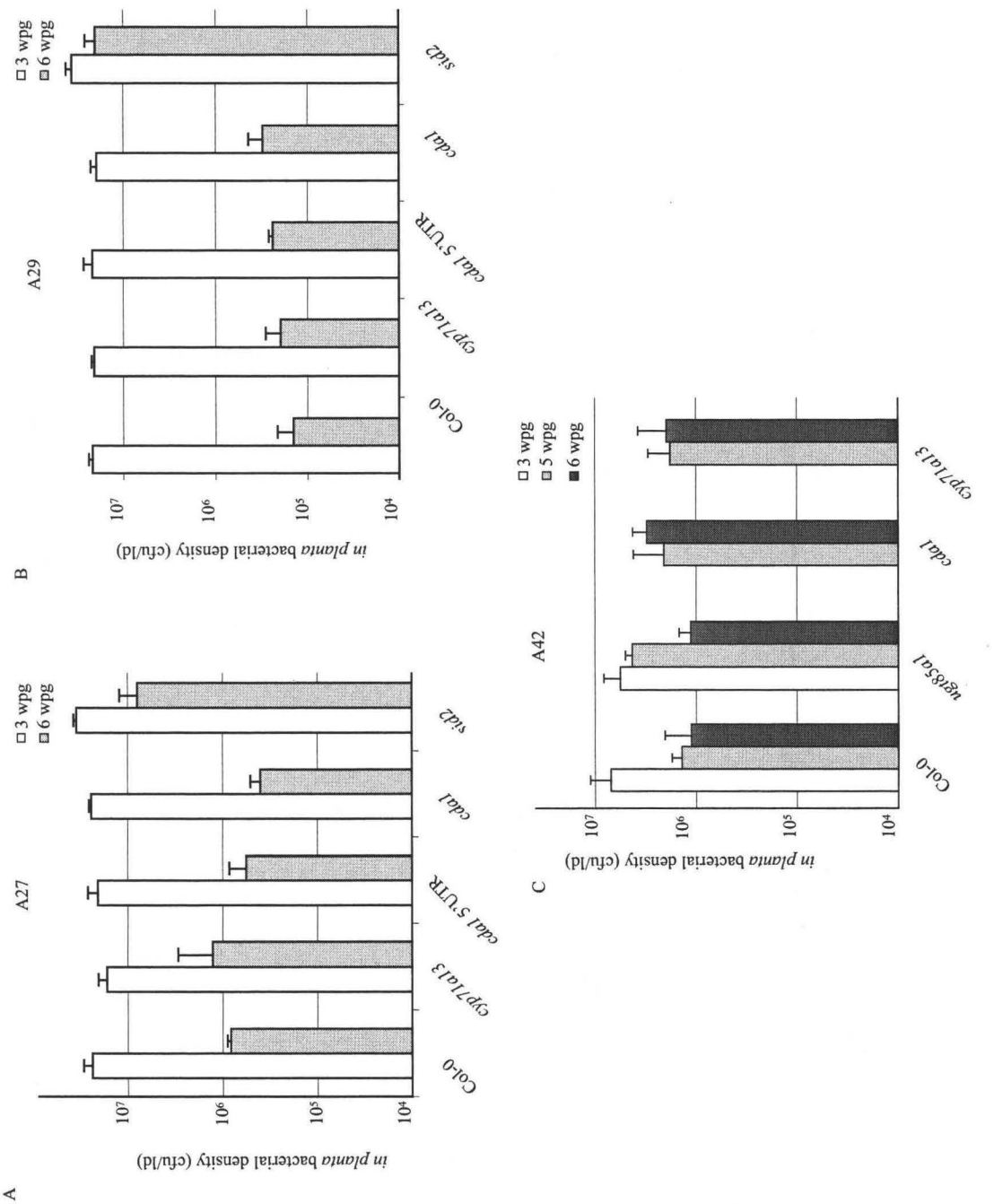

A
A27
□ 3 wpg
■ 6 wpg
in planta bacterial density (cfu/ld)
10^7
10^6
10^5
10^4
Col-0
cyp71a13
cdaI 5'UTR
cdaI
sid2
B
A29
□ 3 wpg
■ 6 wpg
in planta bacterial density (cfu/ld)
Col-0
cyp71a13
cdaI 5'UTR
cdaI
sid2
C
A42
□ 3 wpg
□ 5 wpg
■ 6 wpg
in planta bacterial density (cfu/ld)
Col-0
ugt63a1
cdaI
cyp71a13

Fig A3: *In planta Pst* growth (logarithmic scale) in Col-0, *lox2*, *anac055*, and *sid2* at 3, 4, 5, and 6 wpg. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1. ARR experiments A) A44, B) A46, C) A49, and D) A51 are shown.

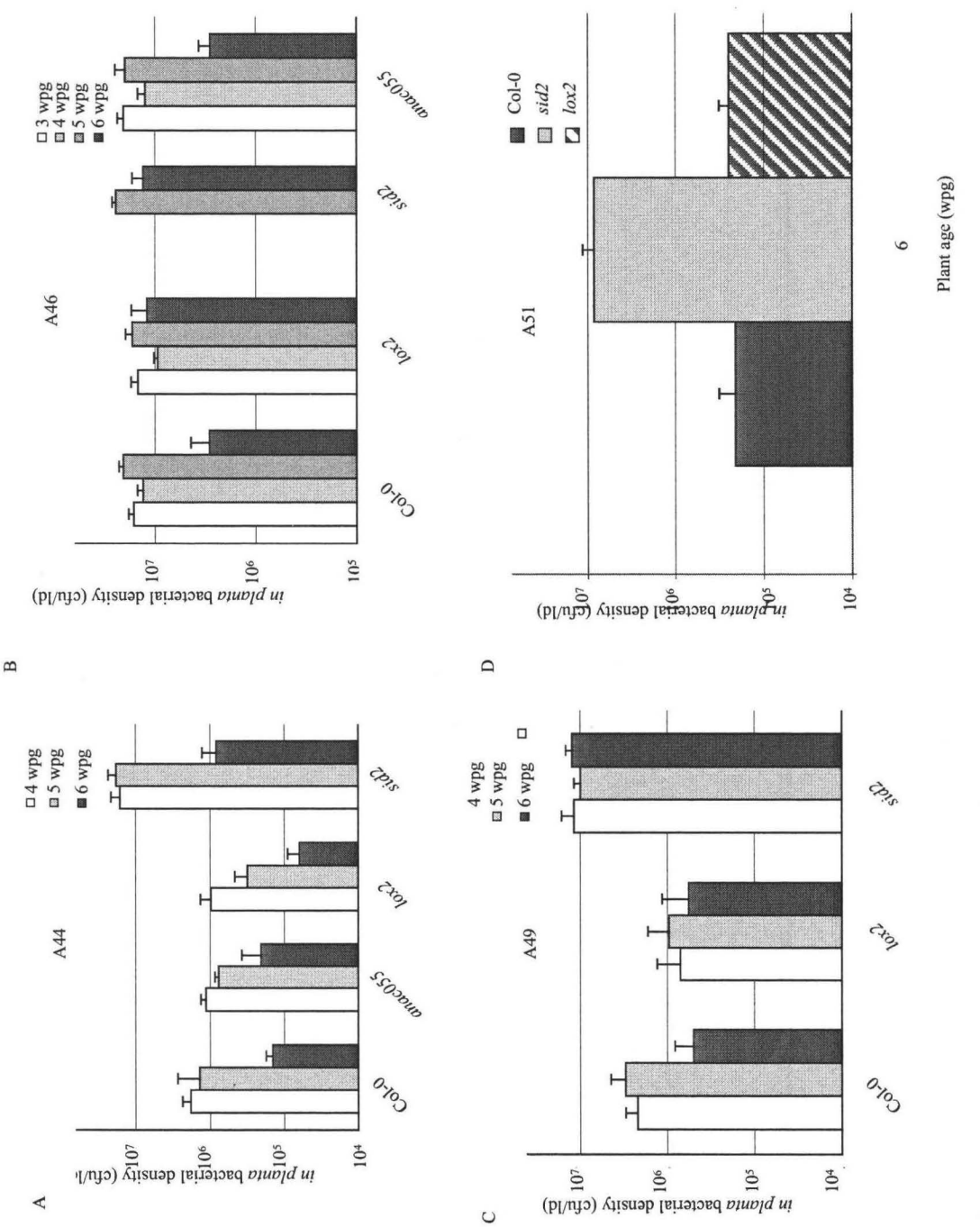
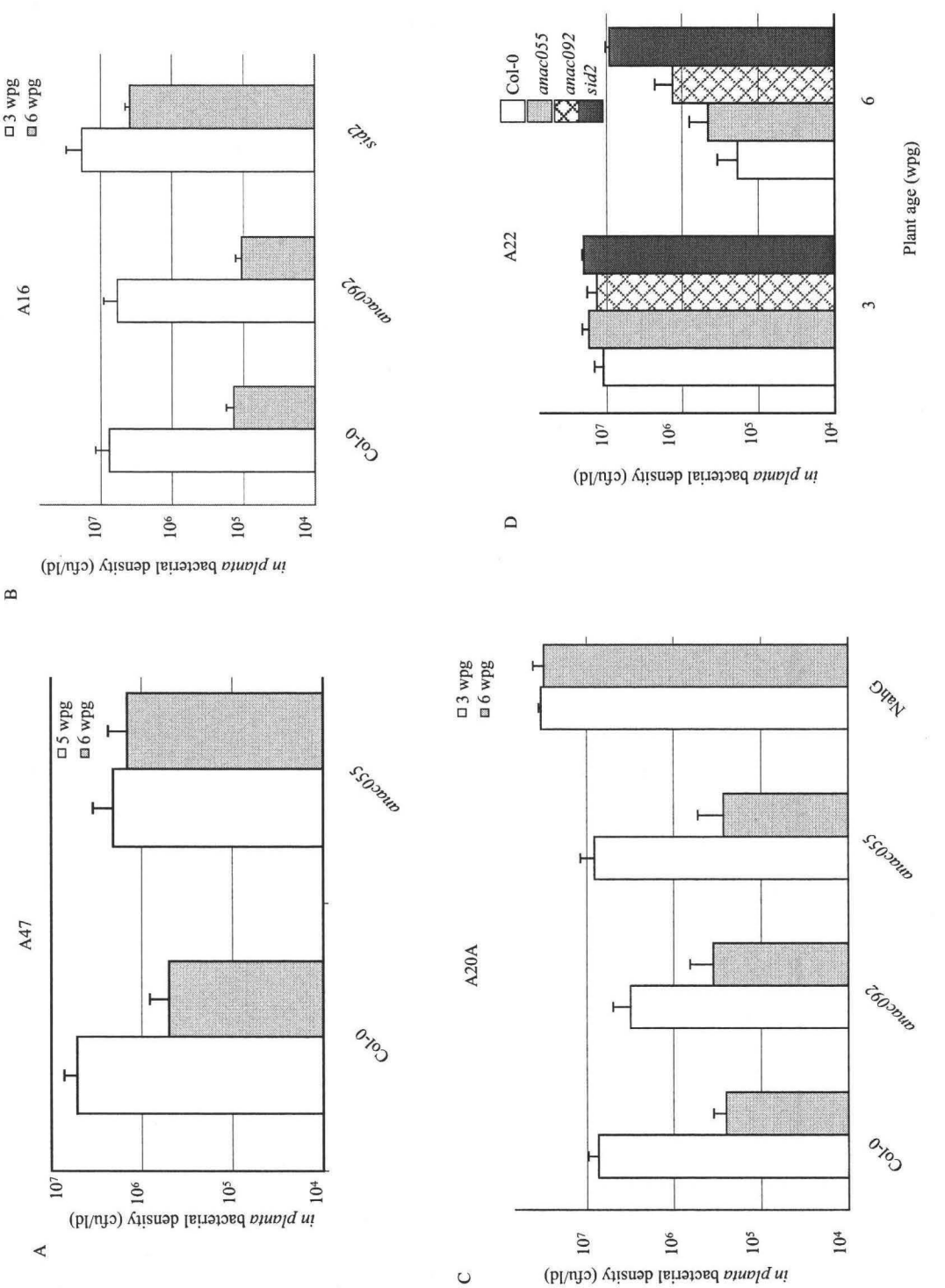

A
B
C
D
A44
A46
A49
A51
4 wpg
5 wpg
6 wpg
3 wpg
Col-0
mac055
lox2
sid2
Col-0
sid2
lox2
in planta bacterial density (cfu/ld)
Plant age (wpg)

Fig A4: *In planta Pst* growth (logarithmic scale) in Col-0, *anac055*, *anac092*, *sid2*, and *NahG* at 3, 4, 5, 6, and 7 wpg. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1. ARR experiments A) A47, B) A16, C)A20A, D) A22, E) A28, and F) A39 are shown.


A
A47
5 wpg
6 wpg
Col-0
anac055
in planta bacterial density (cfu/ld)
B
A16
3 wpg
6 wpg
Col-0
anac092
sid2
in planta bacterial density (cfu/ld)
C
A20A
3 wpg
6 wpg
Col-0
anac092
anac055
NahG
in planta bacterial density (cfu/ld)
D
A22
Col-0
anac055
anac092
sid2
3
6
Plant age (wpg)
in planta bacterial density (cfu/ld)

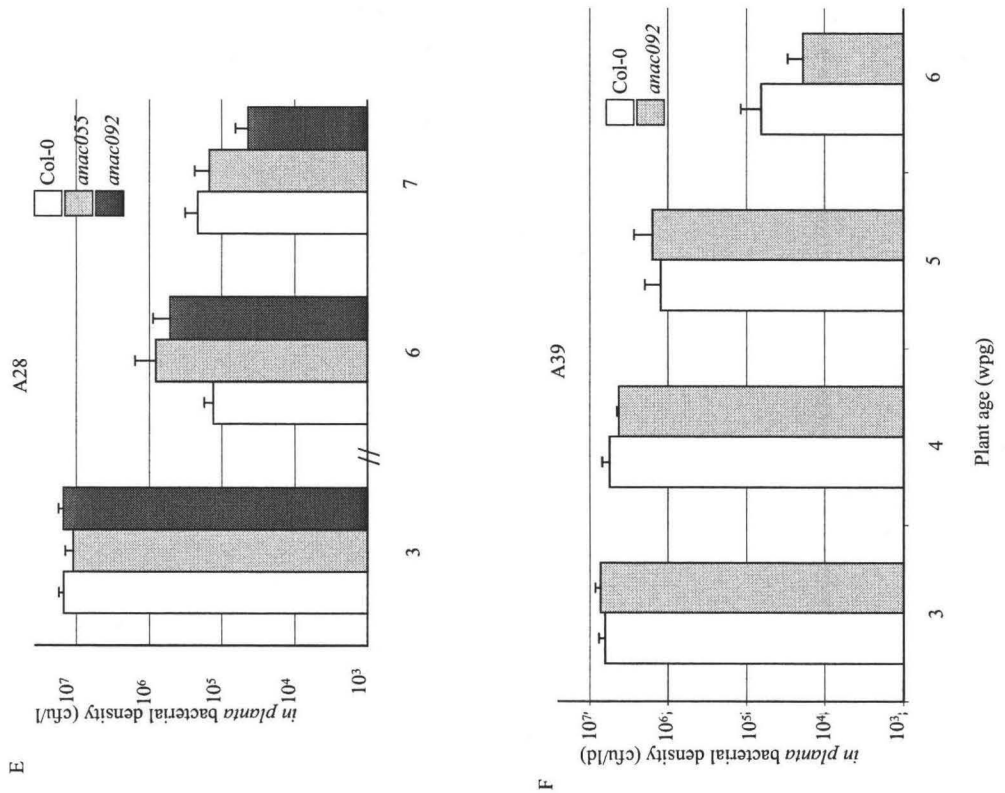
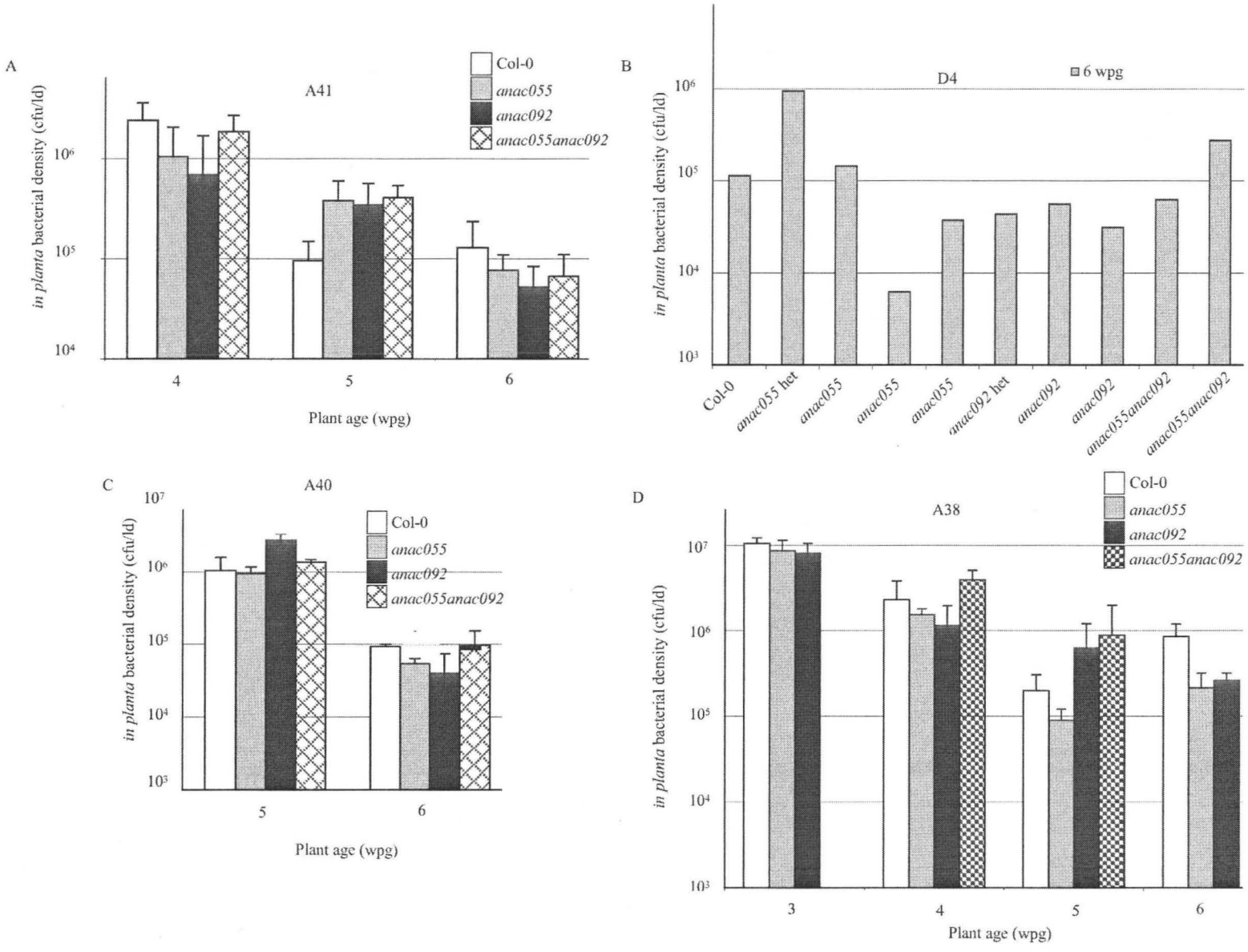

E
A28
Col-0
anac055
anac092
in planta bacterial density (cfu/l
10^7
10^6
10^5
10^4
10^3
3
6
7
F
A39
Col-0
anac092
in planta bacterial density (cfu/ld)
10^7
10^6
10^5
10^4
10^3
3
4
5
6
Plant age (wpg)

Fig A5: *In planta Pst* growth (logarithmic scale) in Col-0, *anac055*, *anac092*, and *anac055anac092* at 3, 4, 5, and 6 wpg. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1. ARR experiments A) A41, B) D4, C) A40, D) A38, and E) A38 are shown. In experiment D4 (B), plants labeled as "het" are heterozygote mutant plants. In experiment A38 (E), plants with macroscopically visible inflorescence stems (flowering) and plants with no macroscopically visible inflorescence stems were analyzed separately.


A
A41
Col-0
anac055
anac092
anac055anac092
in planta bacterial density (cfu/ld)
Plant age (wpg)
B
D4
6 wpg
in planta bacterial density (cfu/ld)
Col-0
anac055 het
anac055
anac055
anac055
anac092 het
anac092
anac092
anac055anac092
anac055anac092
C
A40
Col-0
anac055
anac092
anac055anac092
in planta bacterial density (cfu/ld)
Plant age (wpg)
D
A38
Col-0
anac055
anac092
anac055anac092
in planta bacterial density (cfu/ld)
Plant age (wpg)

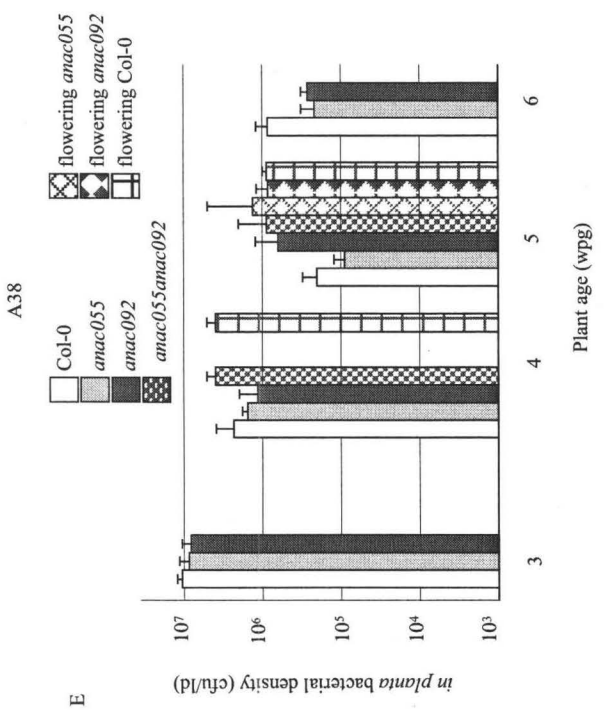

A38
Col-0
anac055
anac092
anac055anac092
flowering anac055
flowering anac092
flowering Col-0
E
in planta bacterial density (cfu/ld)
10^7
10^6
10^5
10^4
10^3
3
4
5
6
Plant age (wpg)

Fig A6: *In planta Pst* growth (logarithmic scale) in Col-0, *anac055*, *anac092*, 35S:*ANAC092*, and *ein2-1* at 4, 5, 6, and 7 wpg. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1. ARR experiments A) A48, B) A50, C) A52, and D) A56 are shown.

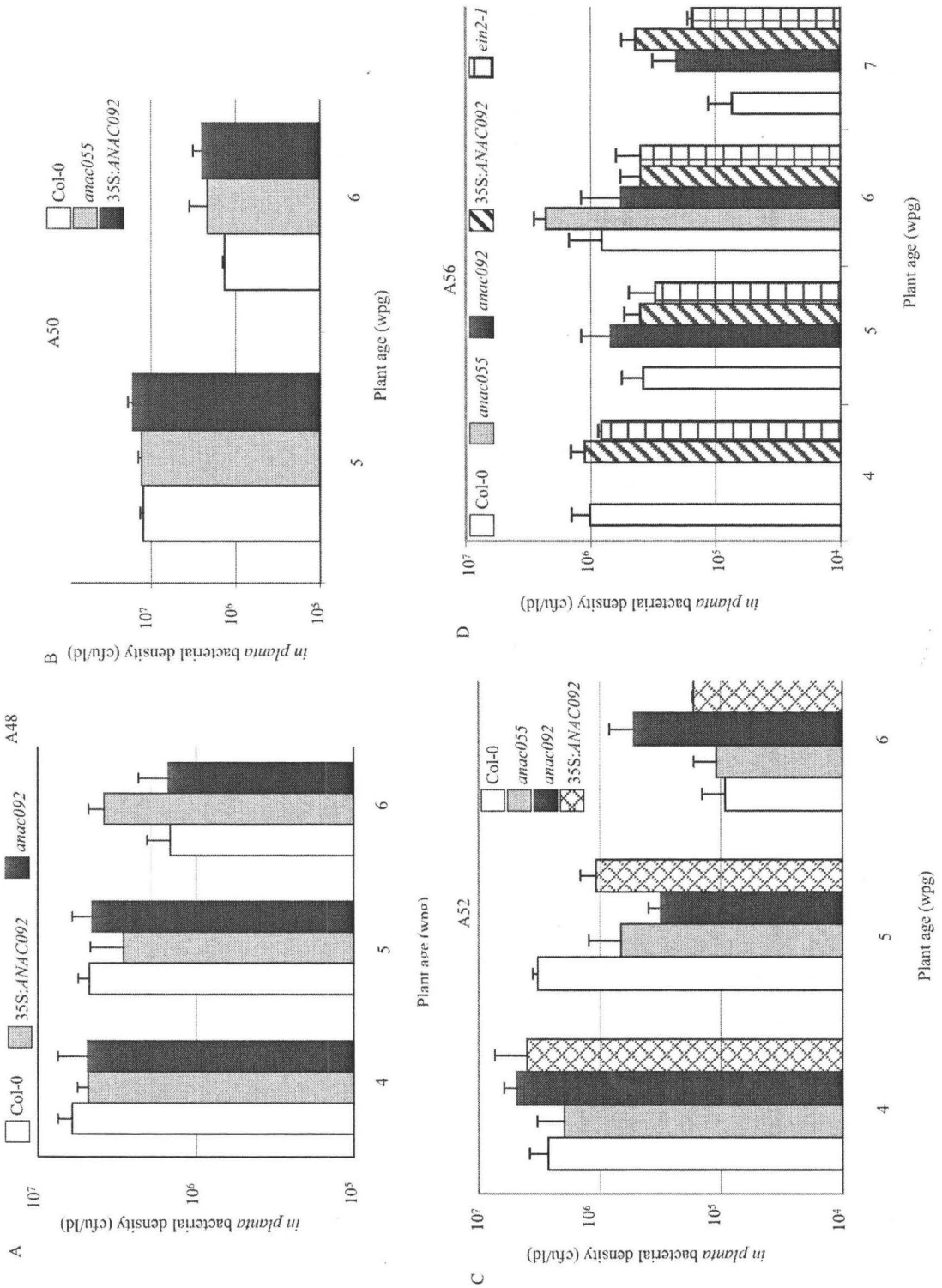

A
B
C
D
A48
A50
A52
A56
Col-0
35S:ANAC092
anac092
anac055
ein2-1
in planta bacterial density (cfu/ld)
Plant age (wpg)

Fig A7: *In planta Pst* growth (logarithmic scale) in 4 week-old Col-0, *anac055*, *anac092*, and *anac055anac092* plants grown under continuous short days (9 hr photoperiod) and plants exposed to long day conditions (16 hr photoperiod) (ARR experiment A41). Long day plants were exposed to at least three long days at 3 wpg and thereafter returned to short day conditions. Plants were inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.

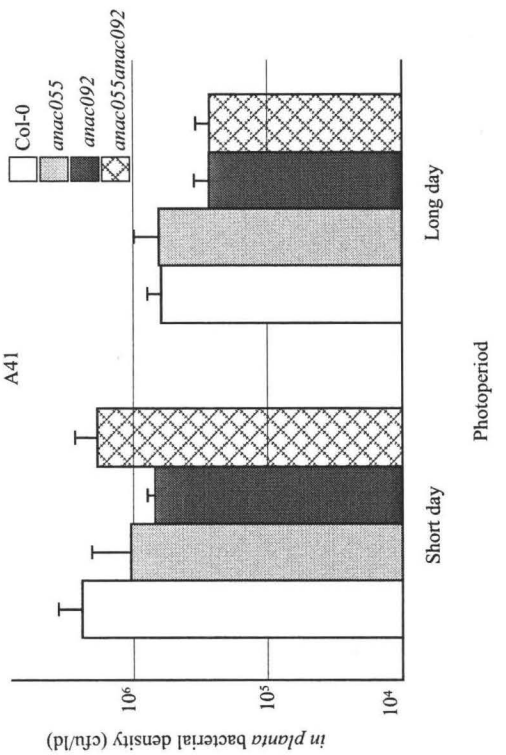
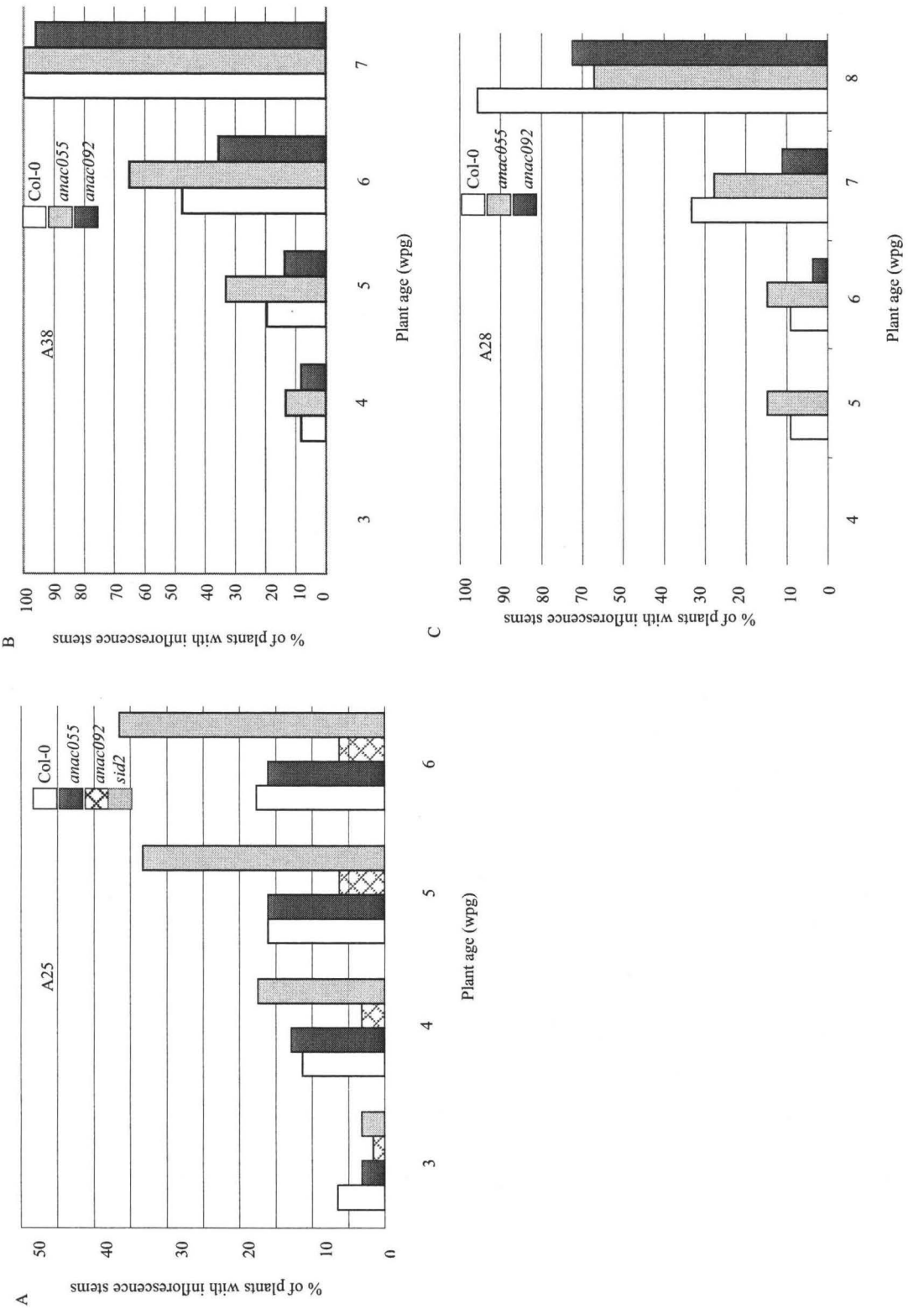

Col-0
anac055
anac092
anac055anac092
A41
Long day
Short day
Photoperiod
10^6
10^5
10^4
in planta bacterial density (cfu/ld)

Fig A8: Percent of plants with macroscopically visible inflorescence stems for Col-0, *anac092*, *anac055*, and 35S:*ANAC092* during ARR experiments A) A25, B) A38, C) A28, D) A50, E) A48, F) A52, and G) A56 at 3, 4, 5, 6, 7, and 8 wpg.


A
% of plants with inflorescence stems
Plant age (wpg)
A25
Col-0
anac055
anac092
sid2
B
% of plants with inflorescence stems
Plant age (wpg)
A38
Col-0
anac055
anac092
C
% of plants with inflorescence stems
Plant age (wpg)
A28
Col-0
anac055
anac092

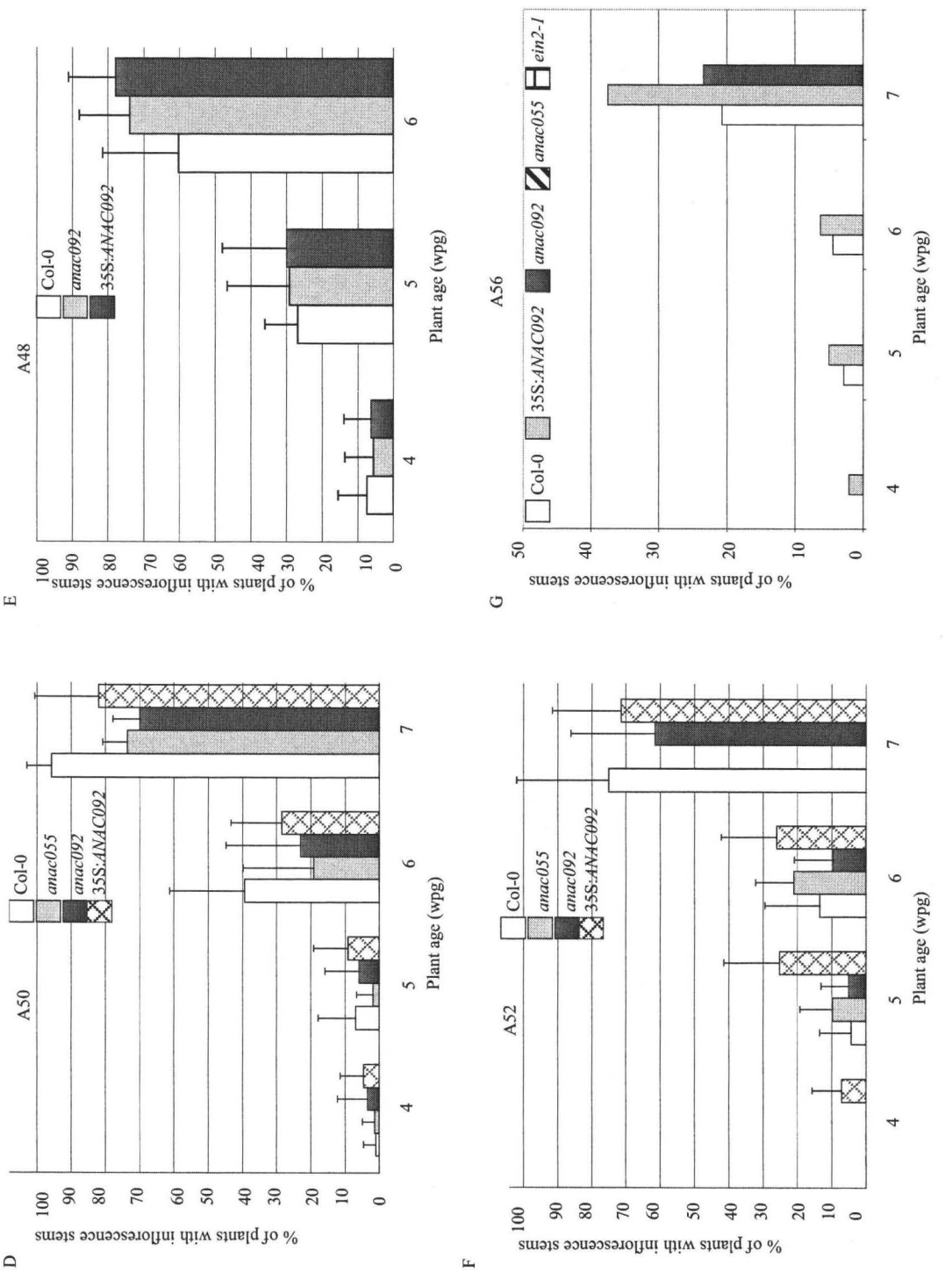
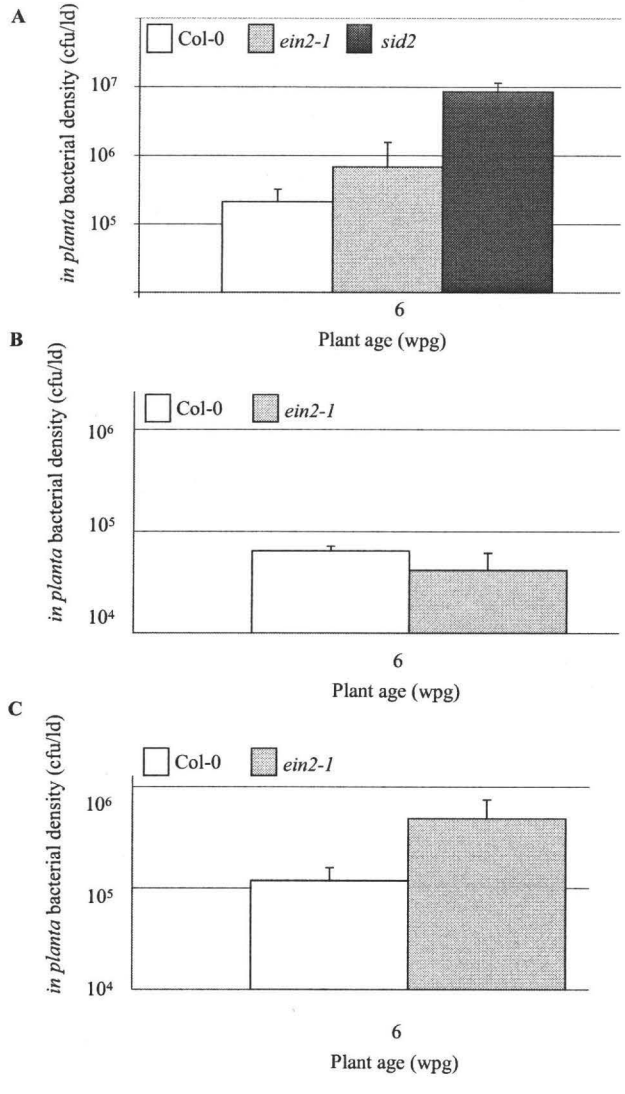

D
A50
Col-0
anac055
anac092
35S:ANAC092
% of plants with inflorescence stems
Plant age (wpg)
E
A48
Col-0
anac092
35S:ANAC092
% of plants with inflorescence stems
Plant age (wpg)
F
A52
Col-0
anac055
anac092
35S:ANAC092
% of plants with inflorescence stems
Plant age (wpg)
G
A56
Col-0
35S:ANAC092
anac092
anac055
ein2-1
% of plants with inflorescence stems
Plant age (wpg)

Fig A9: *In planta Pst* growth in Col-0 and *ein2-1* (6 wpg), and RT-PCR analysis of *ANAC055* and *ANAC092* in mature (6 wpg) Col-0 and *ein2-1* from ARR experiments A51, A54, and A55. *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.


A
Col-0
ein2-1
sid2
in planta bacterial density (cfu/ld)
10^7
10^6
10^5
6
Plant age (wpg)
B
Col-0
ein2-1
in planta bacterial density (cfu/ld)
10^6
10^5
10^4
6
Plant age (wpg)
C
Col-0
ein2-1
in planta bacterial density (cfu/ld)
10^6
10^5
10^4
6
Plant age (wpg)

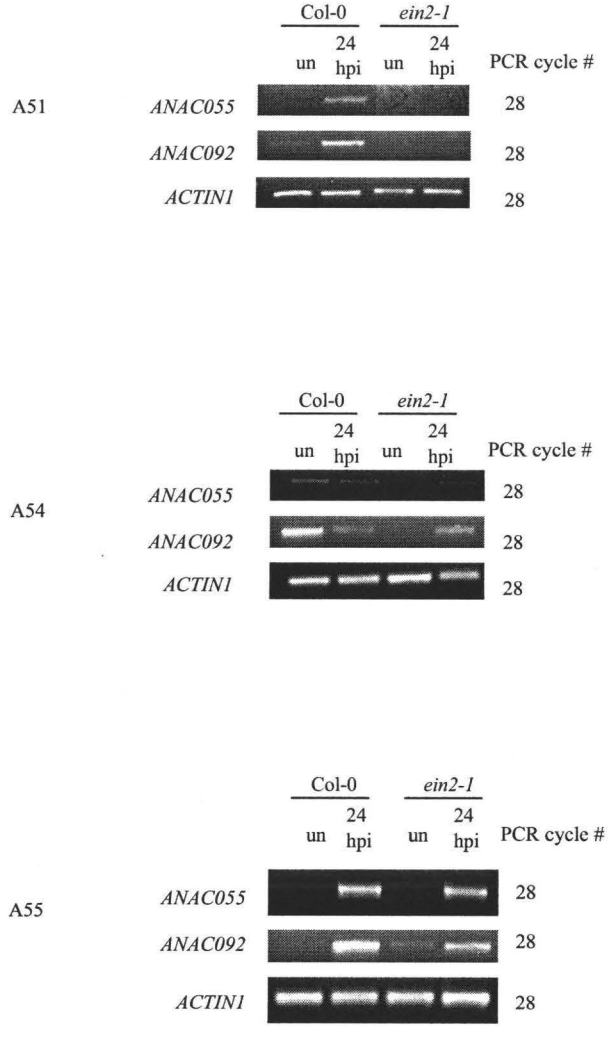

A51
Col-0
ein2-1
un
24 hpi
un
24 hpi
PCR cycle #
ANAC055
28
ANAC092
28
ACTIN1
28
A54
Col-0
ein2-1
un
24 hpi
un
24 hpi
PCR cycle #
ANAC055
28
ANAC092
28
ACTIN1
28
A55
Col-0
ein2-1
un
24 hpi
un
24 hpi
PCR cycle #
ANAC055
28
ANAC092
28
ACTIN1
28

Fig A10: *In planta Pst* growth (logarithmic scale) in young (3 wpg) and mature (6 wpg) T-DNA insertion mutant plants of some microarray genes (*β-gal4*, *ugt85a1*, *cda1*, *glycerol-3-transporter*, *anac055*), *lra* (*HSP90*), wild-type, *sid2*, and *NahG* plants (ARR experiment A5). RT-PCR analysis of *ICS1* and *PDF1.2a* expression in mature (6 wpg) Col-0 and *anac055* at 24 hpi. *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 1010^{6} cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.

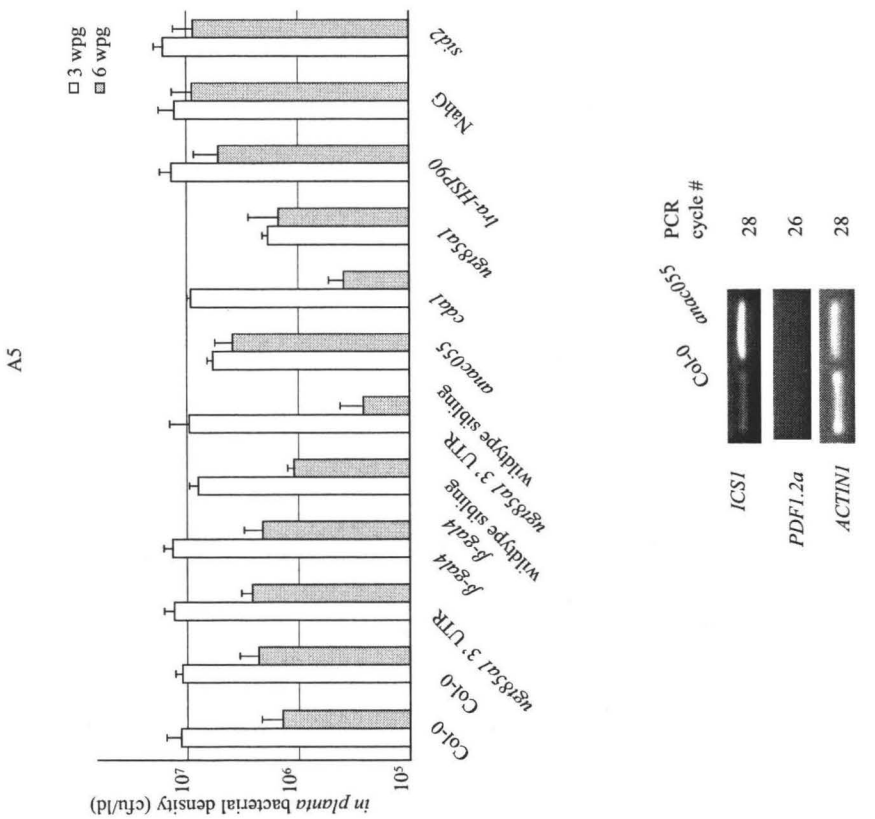
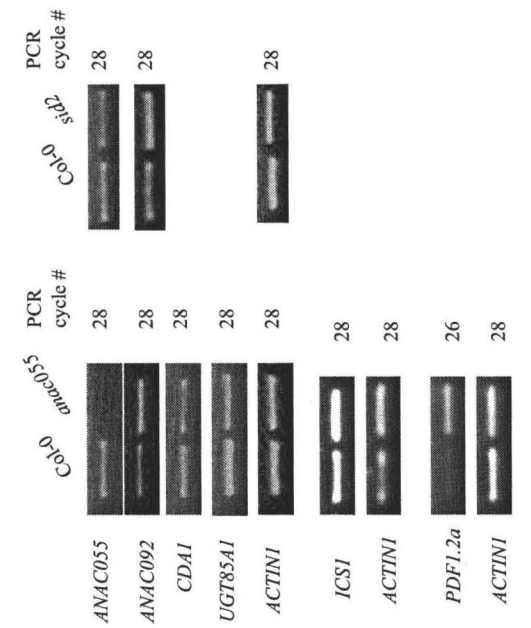

A5
□ 3 wpg
■ 6 wpg
in planta bacterial density (cfu/ld)
10^7
10^6
10^5
Col-0
Col-0
ugt85a1 3' UTR
fls-gal4
fls-gal4
wildtype sibling
ugt85a1 3' UTR
wildtype sibling
amec055
cda1
ugt85a1
tra-HSP90
NahG
sid2
Col-0
amec055
PCR cycle #
28
26
28
ICS1
PDF1.2a
ACTIN1

Fig A11: *In planta Pst* growth (logarithmic scale) in young (3 wpg) and mature (6 wpg) T-DNA insertion mutant plants of some microarray genes (*anac055, cyp71a13*, *cda1*, *glycerol-3-transporter*, *ugt85a1*, *anac092*, *β-gal4*), wild-type, *sid2*, and *NahG* plants (ARR experiment A12). RT-PCR analysis of *ANAC055*, *ANAC092*, *UGT85A1*, *CDA1*, *ICS1* and *PDF1.2a* expression in mature (6 wpg) Col-0, *anac055*, and *sid2* at 24 hpi. *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.


Col-0
anac055
PCR cycle #
28
28
28
28
28
28
28
26
28
ANAC055
ANAC092
CDA1
UGT85A1
ACTIN1
ICS1
ACTIN1
PDF1.2a
ACTIN1
Col-0
sid2
PCR cycle #
28
28
28

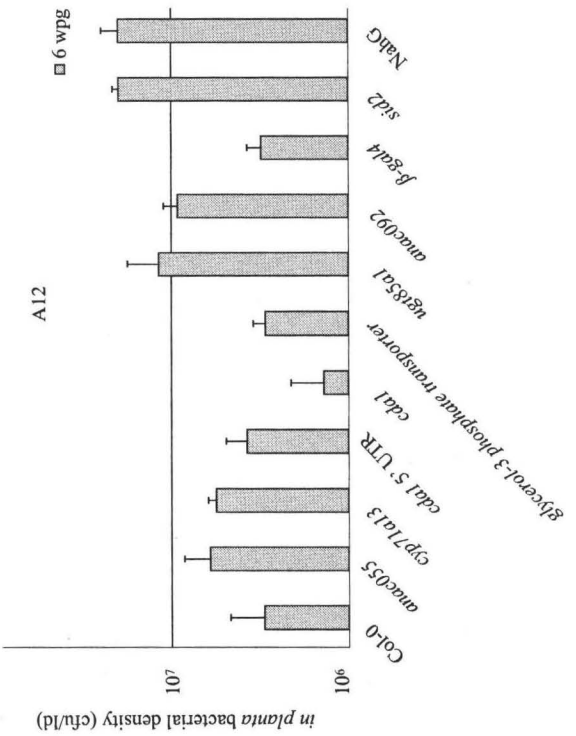
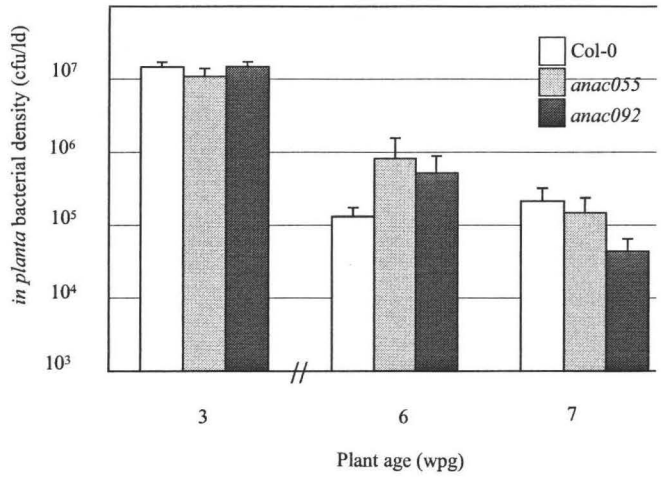
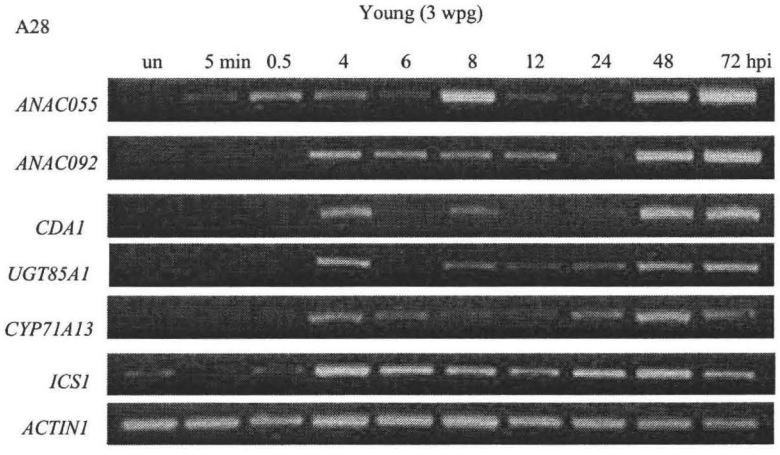

A12
6 wpg
Col-0
anac055
cyp71a13
cdu1 5' UTR
cdu1
glycerol-3 phosphate transporter
ugt85a1
anac092
β-gal4
sid2
NahG
10^7
10^6
in planta bacterial density (cfu/ld)

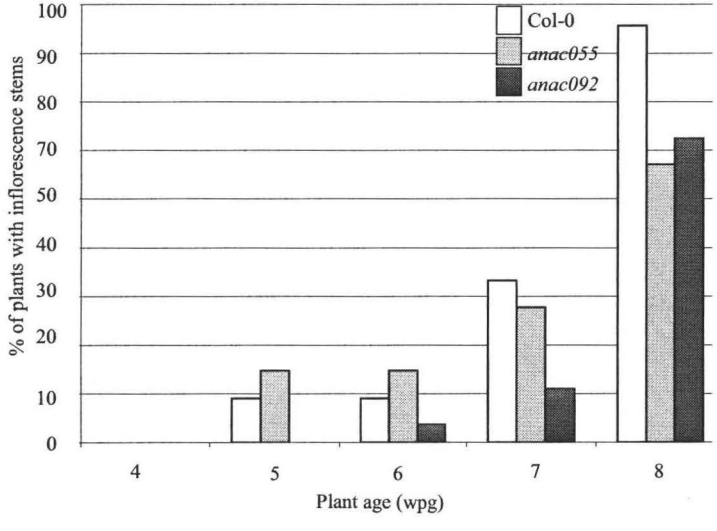
Fig A12: *In planta Pst* growth (logarithmic scale) in young (3 wpg) and mature (6 wpg) Col-0, *anac055*, *anac092*, and *sid2* (ARR experiment A22). RT-PCR analysis of *ANAC092* expression in mock-inoculated leaves (10 mM $MgCl_2$) and leaves inoculated with *Pst* collected from mature Col-0 (6 wpg) at 6 and 24 hpi. RT-PCR analysis of *ANAC055, ANAC092*, *UGT85A1*, *CDA1*, *CYP71A13*, and *ICS1* expression in mature (6 wpg) Col-0, *anac055*, *anac092*, and *sid2* at 24 hpi with *Pst*. RT-PCR analysis of *PR1*, *LOX2*, and *PDF1.2a* expression in mature (6 wpg) Col-0 and *anac055* at 24 hpi with *Pst*. *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.

A22 15% Col-0 flowering at 6 wpg

Fig A13: *In planta Pst* growth (logarithmic scale) in young (3 wpg) and mature (6 and 7 wpg) Col-0, *anac055*, and *anac092* (ARR experiment A28). The percent of Col-0, *anac055*, and *anac092* plants with macroscopically visible inflorescence stems at 4, 5, 6, 7, and 8 wpg is presented. RT-PCR analysis of *ANAC055, ANAC092*, *UGT85A1*, *CDA1*, *CYP71A13*, and *ICS1* expression in young (3 wpg) and mature (6 wpg) Col-0 at different time point after *Pst* inoculation. *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.


in planta bacterial density (cfu/ld)
10⁷
10⁶
10⁵
10⁴
10³
Col-0
anac055
anac092
3
6
7
Plant age (wpg)


A28
Young (3 wpg)
un
5 min
0.5
4
6
8
12
24
48
72 hpi
ANAC055
ANAC092
CDA1
UGT85A1
CYP71A13
ICS1
ACTIN1


% of plants with inflorescence stems
100
90
80
70
50
40
30
20
10
0
Col-0
anac055
anac092
4
5
6
7
8
Plant age (wpg)

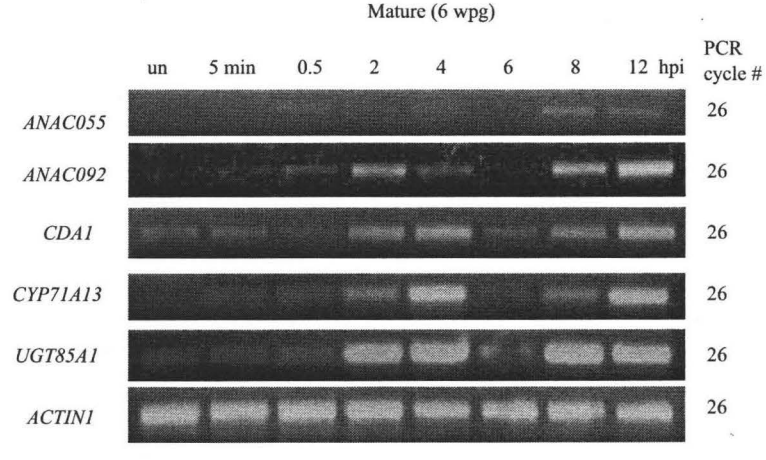

Mature (6 wpg)
un
5 min
0.5
2
4
6
8
12 hpi
PCR cycle #
ANAC055
ANAC092
CDA1
CYP71A13
UGT85A1
ACTIN1
26
26
26
26
26
26

Fig A14: RT-PCR analysis of *ANAC055*, *ANAC092*, *CDA1*, *UGT85A1*, *CYP71A13*, and *LOX2* expression in mock-inoculated leaves (10 mM $MgCl_2$) and leaves inoculated with *Pst* collected from young (3 wpg) and mature Col-0 (6 wpg) at different time points post inoculation (ARR experiments A28 and A29). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000.

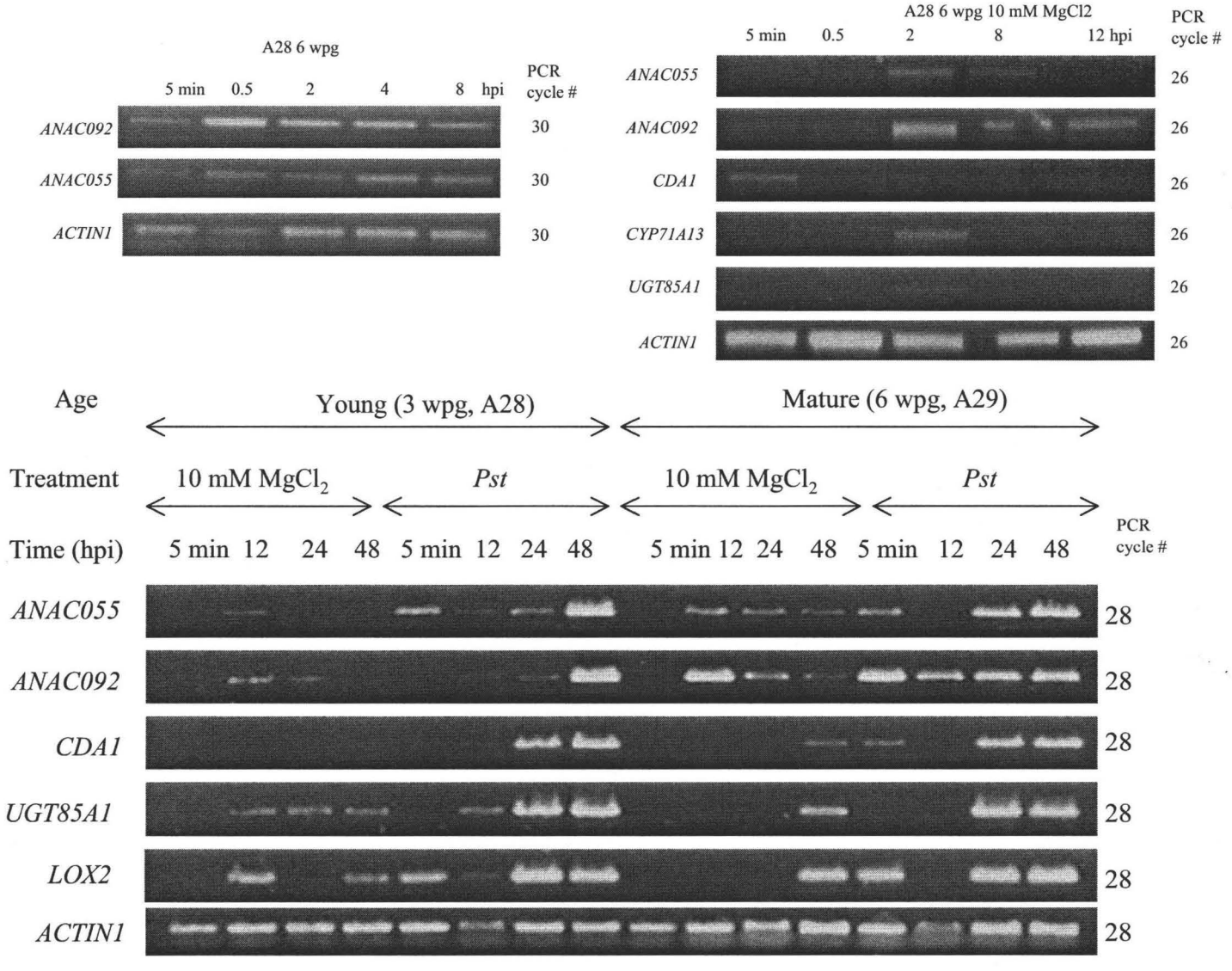
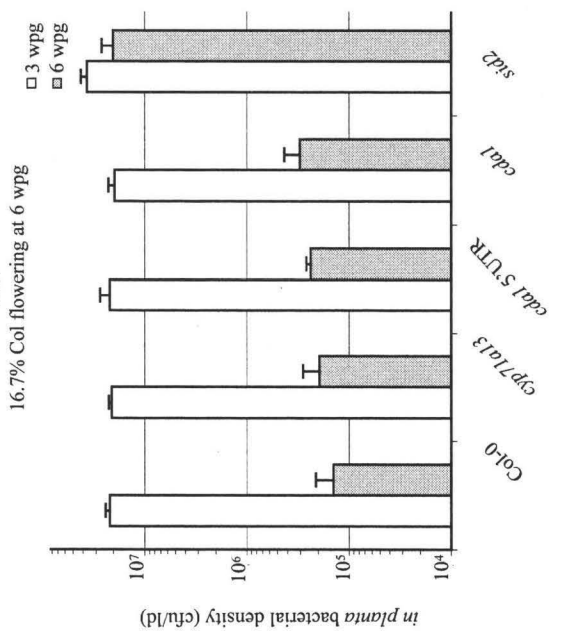

A28 6 wpg
5 min 0.5 2 4 8 hpi
PCR cycle #
ANAC092 30
ANAC055 30
ACTIN1 30
A28 6 wpg 10 mM MgCl2
5 min 0.5 2 8 12 hpi
PCR cycle #
ANAC055 26
ANAC092 26
CDA1 26
CYP71A13 26
UGT85A1 26
ACTIN1 26
Age
Young (3 wpg, A28)
Mature (6 wpg, A29)
Treatment
10 mM MgCl2
Pst
10 mM MgCl2
Pst
Time (hpi)
5 min 12 24 48 5 min 12 24 48 5 min 12 24 48 5 min 12 24 48
PCR cycle #
ANAC055 28
ANAC092 28
CDA1 28
UGT85A1 28
LOX2 28
ACTIN1 28

Fig A15: *In planta Pst* growth (logarithmic scale) in young (3 wpg) and mature (6 wpg) Col-0, *cyp71a13*, *cda1*, and *sid2* (ARR experiment A29). RT-PCR analysis of *ANAC055, ANAC092*, *CDA1*, and *UGT85A1* expression in mock-inoculated leaves (10 mM $MgCl_2$) and leaves inoculated with *Pst* collected from mature Col-0 (6 wpg) at different time points post inoculation. *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.


A29
16.7% Col flowering at 6 wpg
□ 3 wpg
■ 6 wpg
in planta bacterial density (cfu/ld)
10^7
10^6
10^5
10^4
Col-0
cyp71a13
cdaI 5'UTR
cdaI
sid2

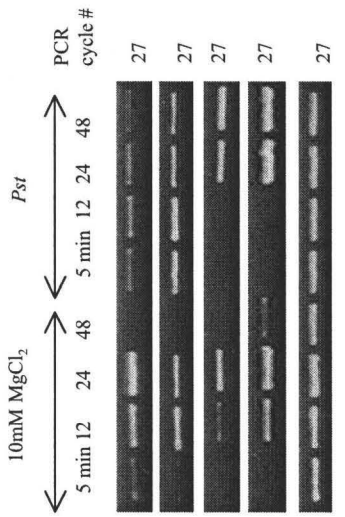
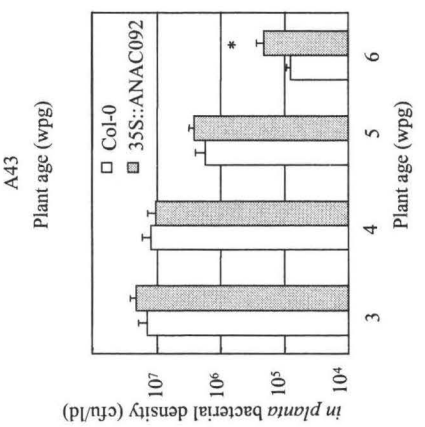

10mM MgCl2
Pst
PCR cycle #
5 min 12 24 48 5 min 12 24 48
Time (hpi)
ANAC055
ANAC092
CDA1
UGT85A1
ACTIN1
27
27
27
27
27

Fig A16: *In planta Pst* growth (logarithmic scale) in Col-0 and 35S:*ANAC092* at 3, 4, 5, and 6 wpg (ARR experiment A43). RT-PCR analysis of *ANAC055, ANAC092*, *CDA1*, *UGT85A1*, *LOX2*, *PDF1.2a*, *ICS1*, and *PR1* expression in mature (6 wpg) Col-0 and 35S:*ANAC092* (untreated and at 24 hpi). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.


A43
Plant age (wpg)
Col-0
35S::ANAC092
*
in planta bacterial density (cfu/ld)
10^7
10^6
10^5
10^4
3
4
5
6
Plant age (wpg)

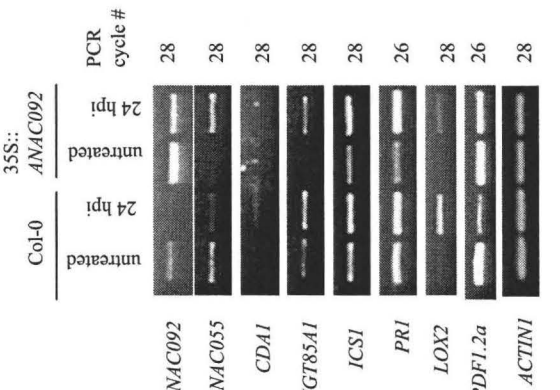

35S::
ANAC092
Col-0
untreated
24 hpi
untreated
24 hpi
PCR
cycle #
ANAC092
ANAC055
CDA1
UGT85A1
ICS1
PR1
LOX2
PDF1.2a
ACTIN1
28
28
28
28
28
26
28
26
28

Fig A17: *In planta Pst* growth (logarithmic scale) in Col-0, *anac055*, *lox2*, and *sid2* at 4, 5, and 6 wpg (ARR experiment A44). RT-PCR analysis of *ANAC055* and *ANAC092* expression in mature (6 wpg) Col-0, *lox2*, and *sid2* (untreated and at 24 hpi). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.

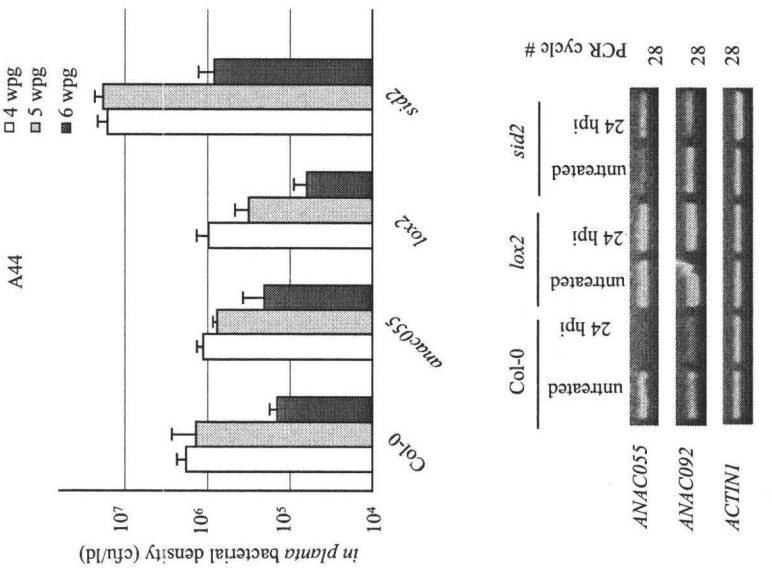

A44
□ 4 wpg
▫ 5 wpg
■ 6 wpg
in planta bacterial density (cfu/ld)
10^7
10^6
10^5
10^4
Col-0
anac055
lox2
sid2
Col-0
untreated
24 hpi
lox2
untreated
24 hpi
sid2
untreated
24 hpi
PCR cycle #
28
28
28
ANAC055
ANAC092
ACTIN1

Fig A18: *In planta Pst* growth (logarithmic scale) in Col-0, *anac055*, *lox2*, and *sid2* at 3, 4, 5, and 6 wpg (ARR experiment A46). RT-PCR analysis of *ANAC055*, *ANAC092*, *CDA1*, *UGT85A1* expression in mature (6 wpg) Col-0, *lox2*, and *sid2*. Leaf samples were collected from untreated plants, wounded leaves (needless syringe pressed down on leaf to damage tissue but not break through the leaf), mock-inoculated plants (10 mM $MgCl_2$), and plants inoculated with *Pst* DC3000 and *Pst* DC3118 (coronatine-deficient). All samples were collected at 24 hrs after treatment. *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} *Pst*. For details of *Pst* quantification refer to the legend of Fig 3.1.

A46
3 wpg
4 wpg
5 wpg
6 wpg
in planta bacterial density (cfu/ld)
10⁷
10⁶
10⁵
Col-0
lox2
sid2
anac055
Col-0
lox2
sid2
untreated
10 mM MgCl2
wounded
Pst DC3000
Pst DC3118
untreated
Pst DC3000
Pst DC3118
untreated
Pst DC3000
PCR cycle #
ANAC055
28
ANAC092
28
CDA1
30
UGT85A1
28
ACTIN1
28

Fig A19: *In planta Pst* growth (logarithmic scale) in Col-0 and *anac055* at 5 and 6 wpg (ARR experiment A47). RT-PCR analysis of *ANAC055*, *ANAC092*, *CDA1*, *UGT85A1*, *ICS1*, *PR1*, *LOX2*, and *PDF1.2a* expression in mature (6 wpg) Col-0 and *anac055* (24 hpi). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.

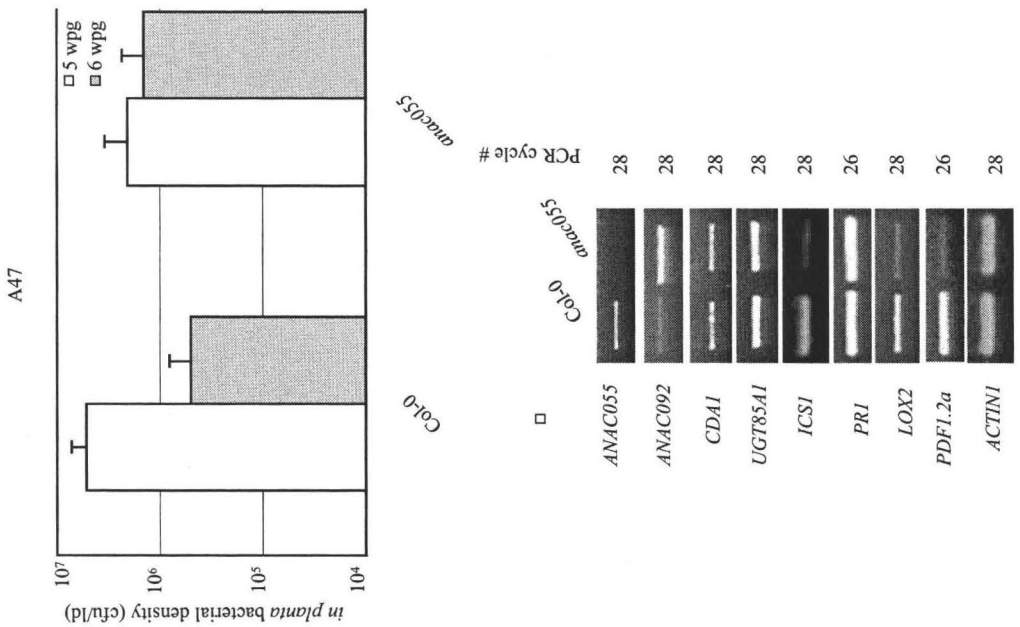

A47
in planta bacterial density (cfu/ld)
10^7
10^6
10^5
10^4
5 wpg
6 wpg
Col-0
anac055
□
Col-0
anac055
PCR cycle #
ANAC055
ANAC092
CDA1
UGT85A1
ICS1
PR1
LOX2
PDF1.2a
ACTIN1
28
28
28
28
28
26
28
26
28

Fig A20: *In planta Pst* growth (logarithmic scale) and flowering time analysis (average of three replicates of percent of plants with macroscopically visible inflorescence stems +/- SD) of Col-0, *anac092*, and 35S:*ANAC092* at 4, 5, and 6 wpg (ARR experiment A48). RT-PCR analysis of *ANAC055, ANAC092*, *CDA1*, *ICS1*, and *PDF1.2a* expression in mature (6 wpg) Col-0 and 35S:*ANAC092* (untreated and at 24 hpi). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.

10^7
10^6
10^5
in planta bacterial density (cfu/ld)
Col-0
35S:*ANAC092*
anac092
4
5
6
Plant age (wpg)

100
90
80
70
60
50
40
30
20
10
0
% of plants with inflorescence stems
Col-0
anac092
35S:*ANAC092*
4
5
6
Plant age (wpg)

A48

Col-0
35S::
ANAC092
untreated
24 hpi
untreated
24 hpi
PCR cycle #
ANAC092 28
ANAC055 28
CDA1 28
ACTIN1 28
ICS1 28
PDF1.2a 26
ACTIN1 28

Fig A21: *In planta Pst* growth (logarithmic scale) in Col-0, *lox2*, and *sid2* at 4, 5, and 6 wpg (ARR experiment A49). RT-PCR analysis of *ANAC055* and *ANAC092* expression in mature (6 wpg) Col-0, *lox2*, and *sid2*. Leaf samples were collected from untreated plants, wounded leaves (needless syringe pressed down on leaf to damage tissue but not break through the leaf), mock-inoculated plants (10 mM $MgCl_2$), and plants inoculated with *Pst* DC3000 and *Pst* DC3118 (coronatine-deficient). All samples were collected at 24 hrs after treatment. *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.

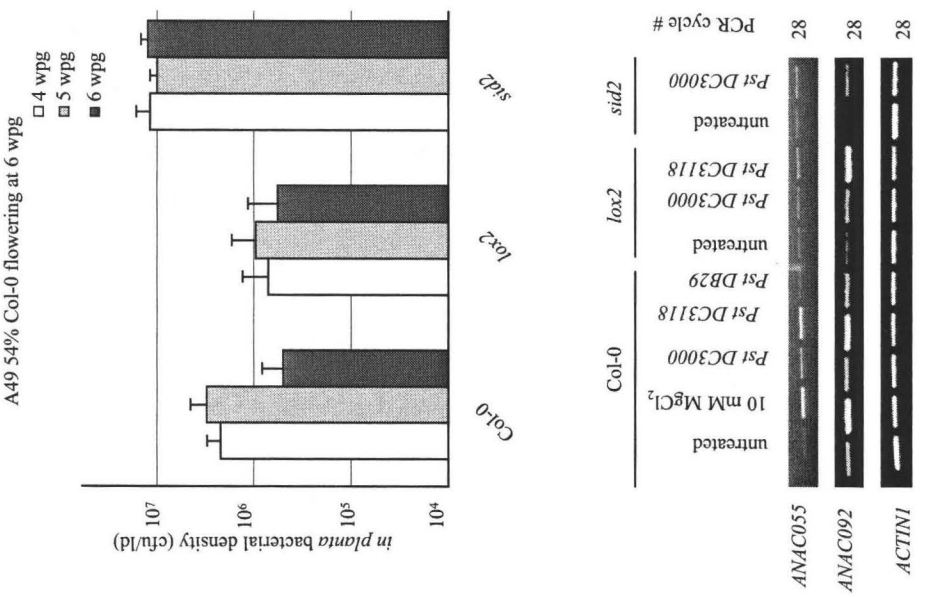

A49 54% Col-0 flowering at 6 wpg
4 wpg
5 wpg
6 wpg
in planta bacterial density (cfu/ld)
10^7
10^6
10^5
10^4
Col-0
lox2
sid2
Col-0
lox2
sid2
untreated
10 mM MgCl2
Pst DC3000
Pst DC3118
Pst DB29
Pst DC3000
Pst DC3118
Pst DC3000
untreated
Pst DC3000
untreated
PCR cycle #
28
28
28
ANAC055
ANAC092
ACTIN1

Fig A22: *In planta Pst* growth (logarithmic scale) and flowering time analysis (average of three replicates of percent of plants with macroscopically visible inflorescence stems +/- SD) of Col-0, *anac055*, and 35S:*ANAC092* at 5 and 6 wpg (ARR experiment A50). RT-PCR analysis of *ANAC055, ANAC092*, *ICS1*, and *PDF1.2a* expression in mature (6 wpg) Col-0, *anac055*, and 35S:*ANAC092* (untreated and at 24 hpi). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.

Col-0
anac055
35S:ANAC092
in planta bacterial density (cfu/ld
10^7
10^6
10^5
5
6
Plant age (wpg)

Col-0
anac055
anac092
35S:ANAC092
% of plants with inflorescence stems
100
90
80
70
60
50
40
30
20
10
0
4
5
6
7
Plant age (wpg)

A50

Col-0
35S::
ANAC092
Col-0
anac055
untreated
24 hpi
untreated
24 hpi
untreated
24 hpi
untreated
24 hpi
PCR cycle #
ANAC055
28
ANAC092
28
ICS1
28
PDF1.2a
26
ACTIN1
28

Fig A23: *In planta Pst* growth (logarithmic scale) in Col-0, *sid2*, *lox2*, *coi1-17*, Ws-2, and *opr3* at 6 wpg (ARR experiment A51). RT-PCR analysis of *ANAC055* and *ANAC092* expression in mature (6 wpg) Col-0, *sid2*, *lox2*, and *coi1-17* (untreated and at 24 hpi). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.

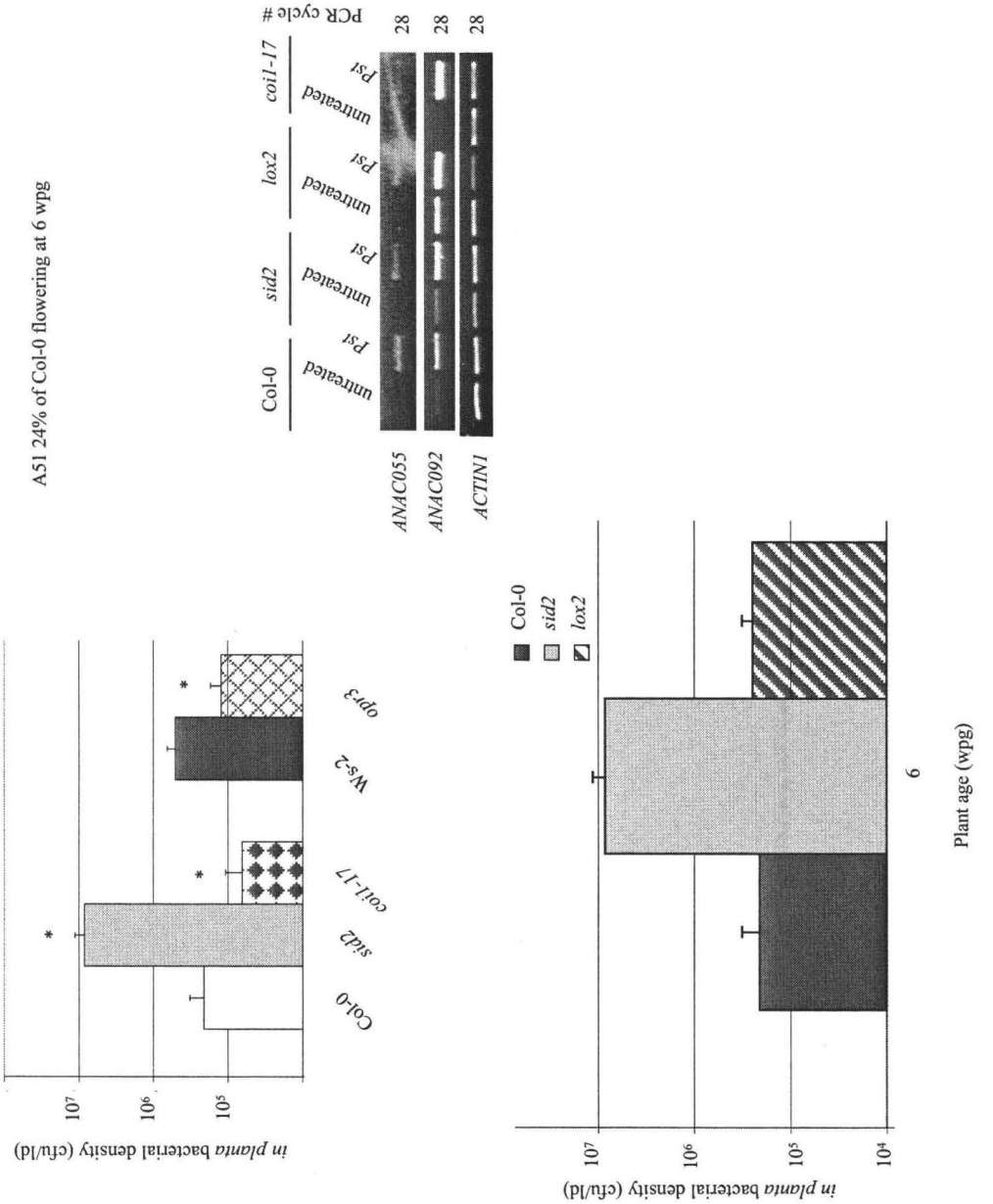
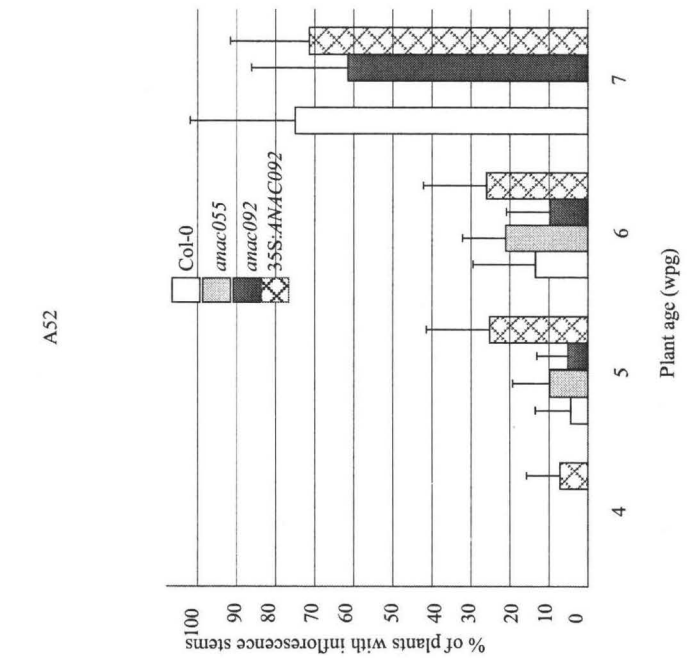
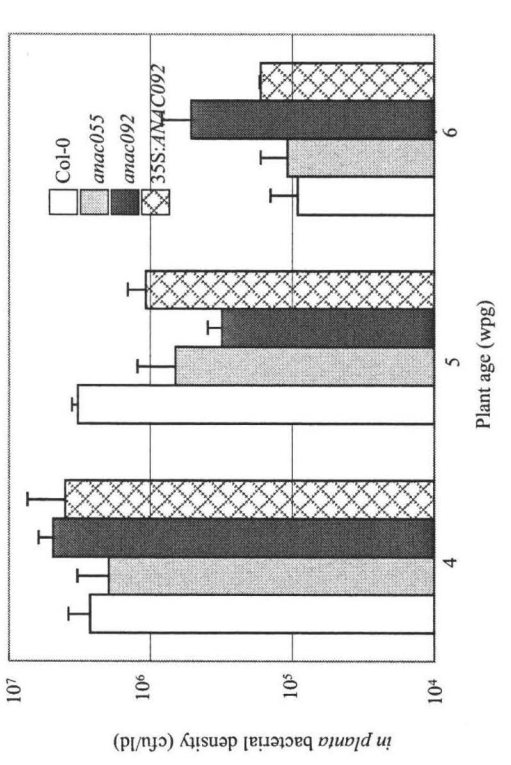

A51 24% of Col-0 flowering at 6 wpg
PCR cycle #
Col-0
sid2
lox2
coi1-17
untreated
Pst
ANAC055
ANAC092
ACTIN1
28
in planta bacterial density (cfu/ld)
Col-0
sid2
coi1-17
Ws-2
opr3
Col-0
sid2
lox2
Plant age (wpg)
6

Fig A24: *In planta Pst* growth (logarithmic scale) and flowering time analysis (average of three replicates of percent of plants with macroscopically visible inflorescence stems +/- SD) of Col-0, *anac055*, *anac092*, and 35S:*ANAC092* at 4, 5, 6, and 7 wpg (ARR experiment A52). RT-PCR analysis of *LOX2*, *PDF1.2a*, *ICS1*, and *PR1* expression in young (4 wpg) and mature (6 wpg) Col-0, and 35S:*ANAC092* (untreated and at 5 min, 12, 24, and 48 hpi). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.


A52
Col-0
anac055
anac092
35S:ANAC092
% of plants with inflorescence stems
0
10
20
30
40
50
60
70
80
90
100
4
5
6
7
Plant age (wpg)


Col-0
anac055
anac092
35S:ANAC092
10^7
10^6
10^5
10^4
4
5
6
Plant age (wpg)
in planta bacterial density (cfu/ld)

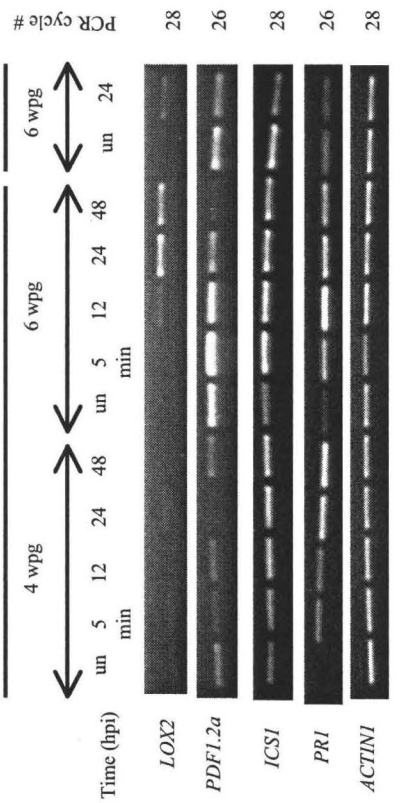
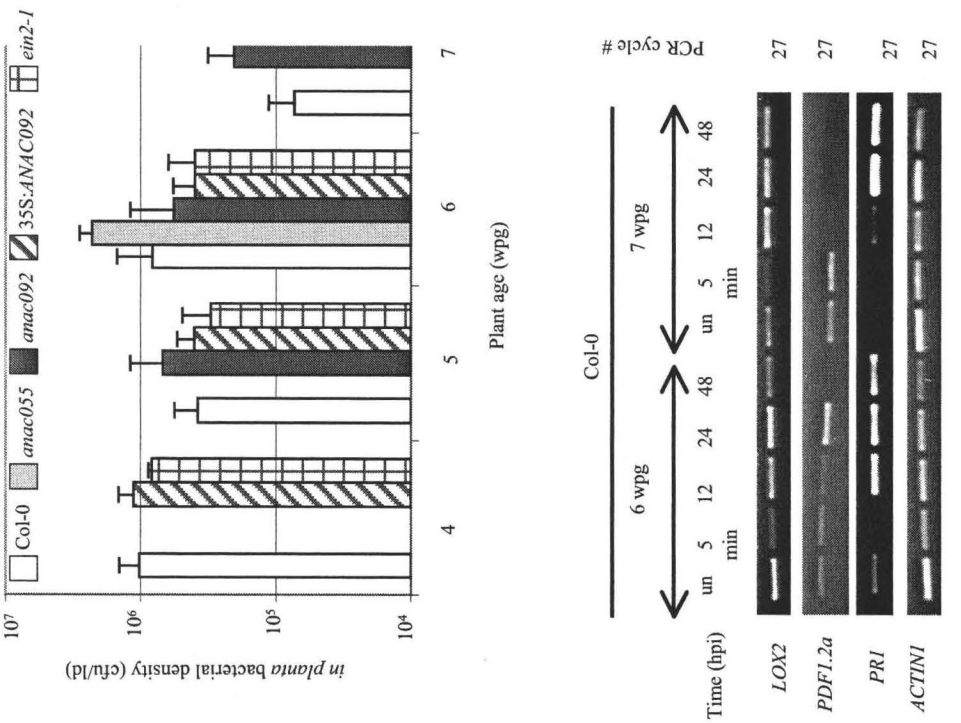

Col-0
35S::ANAC092
4 wpg
6 wpg
6 wpg
Time (hpi)
un
5 min
12
24
48
un
5 min
12
24
48
un
24
PCR cycle #
28
26
28
26
28
LOX2
PDF1.2a
ICS1
PR1
ACTIN1

Fig A25: *In planta Pst* growth (logarithmic scale) and flowering time analysis (average of three replicates of percent of plants with macroscopically visible inflorescence stems +/- SD) of Col-0, *anac055*, *anac092*, 35S:*ANAC092*, and *ein2-1* at 4, 5, 6, and 7 wpg (ARR experiment A56). RT-PCR analysis of *LOX2*, *PDF1.2a*, and *PR1* expression in ARR-incompetent Col-0 plants (6 wpg) and Col-0 plant exhibiting ARR (7 wpg) (untreated and at 5 min, 12, 24, and 48 hpi). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.


Col-0
anac055
anac092
35S:ANAC092
ein2-1
in planta bacterial density (cfu/ld)
Plant age (wpg)
Col-0
6 wpg
7 wpg
Time (hpi)
un 5 12 24 48 un 5 12 24 48
min min
PCR cycle #
LOX2
PDF1.2a
PR1
ACTIN1
27

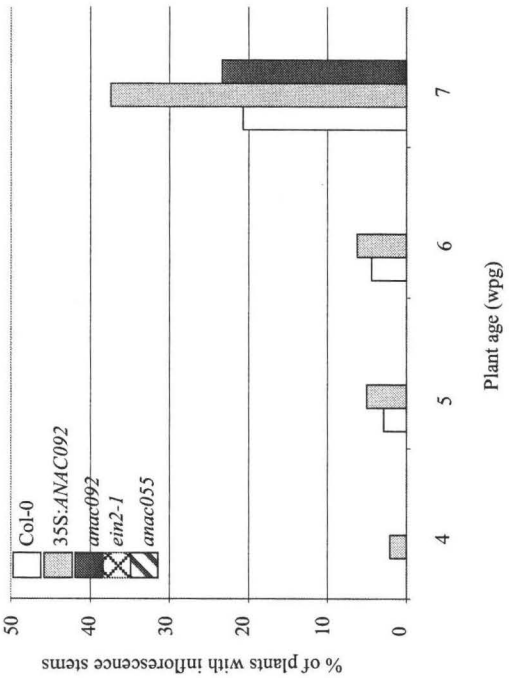
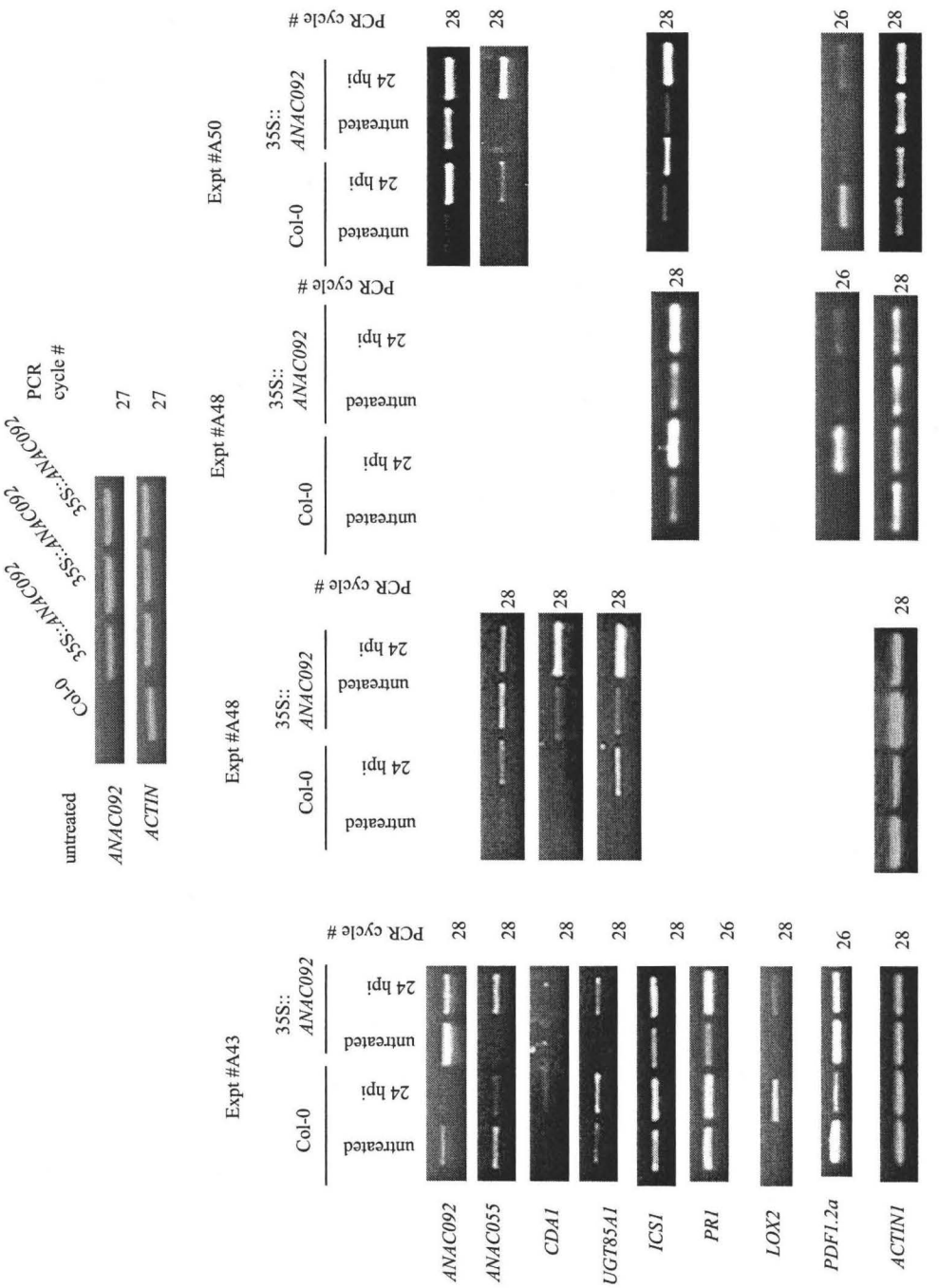

A56
% of plants with inflorescence stems
Col-0
35S:ANAC092
anac092
ein2-1
anac055
Plant age (wpg)

Fig A26: Screening of F1 and F2 generations of the cross between *anac055* and *anac092* for *anac055anac092* plants. Amplification of wild-type *ANAC055* and *ANAC092* and mutant *ANAC055* and *ANAC092* (*anac055* and *anac092*) is indicated. See materials and methods in Chapter 2 for details (Section 3.6c).

ANAC055
anac055
anac092/ANAC092
Col-0
anac092
anac055/ANAC055
Col-0
anac055
anac055/anac055
anac092/anac092
anac055/anac055
anac092/ANAC092
anac055/anac055
anac092/anac092
anac055/ANAC055
anac092/AANC092
anac055/anac055
ANAC092/ANAC092
ANAC055/ANAC055
anac092/ANAC092
ANAC055
anac055
anac092
ANAC092

Fig A27: RT-PCR analysis of *ANAC092*, *ANAC055*, *CDA1*, *UGT85A1*, *ICS1*, *PR1*, *LOX2*, and *PDF1.2a* expression in mature (6 wpg) Col-0 and 35S:*ANAC092* (ARR experiments A43, A48, A50, A52). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000.


Expt #A43
Col-0
35S::ANAC092
untreated
24 hpi
untreated
24 hpi
PCR cycle #
ANAC092
ANAC055
CDA1
UGT85A1
ICS1
PR1
LOX2
PDF1.2a
ACTIN1
28
28
28
28
28
26
28
26
28
Expt #A48
Col-0
35S::ANAC092
untreated
24 hpi
untreated
24 hpi
PCR cycle #
28
28
28
28
28
28
Expt #A48
Col-0
35S::ANAC092
untreated
24 hpi
untreated
24 hpi
PCR cycle #
28
26
28
Expt #A50
Col-0
35S::ANAC092
untreated
24 hpi
untreated
24 hpi
PCR cycle #
28
28
28
26
28
untreated
ANAC092
ACTIN
PCR cycle #
Col-0
35S::ANAC092
35S::ANAC092
35S::ANAC092
27
27

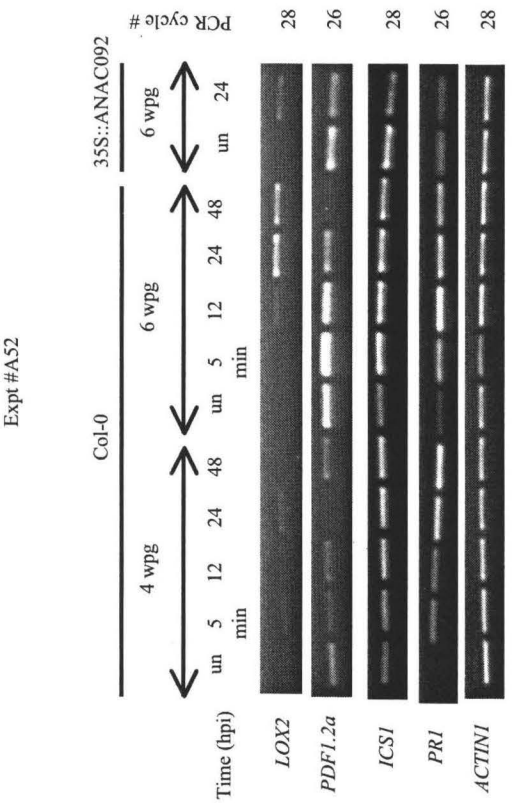

Expt #A52
Col-0
35S::ANAC092
4 wpg
6 wpg
6 wpg
Time (hpi)
un
5 min
12
24
48
un
5 min
12
24
48
un
24
PCR cycle #
LOX2
28
PDF1.2a
26
ICS1
28
PR1
26
ACTIN1
28

Fig A28: RT-PCR analysis of *ANAC092*, *ANAC055*, *CDA1*, *UGT85A1*, *ICS1*, *PR1*, *LOX2*, and *PDF1.2a* expression in mature (6 wpg) Col-0 and *anac055* (ARR experiments A12 and A22). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000.

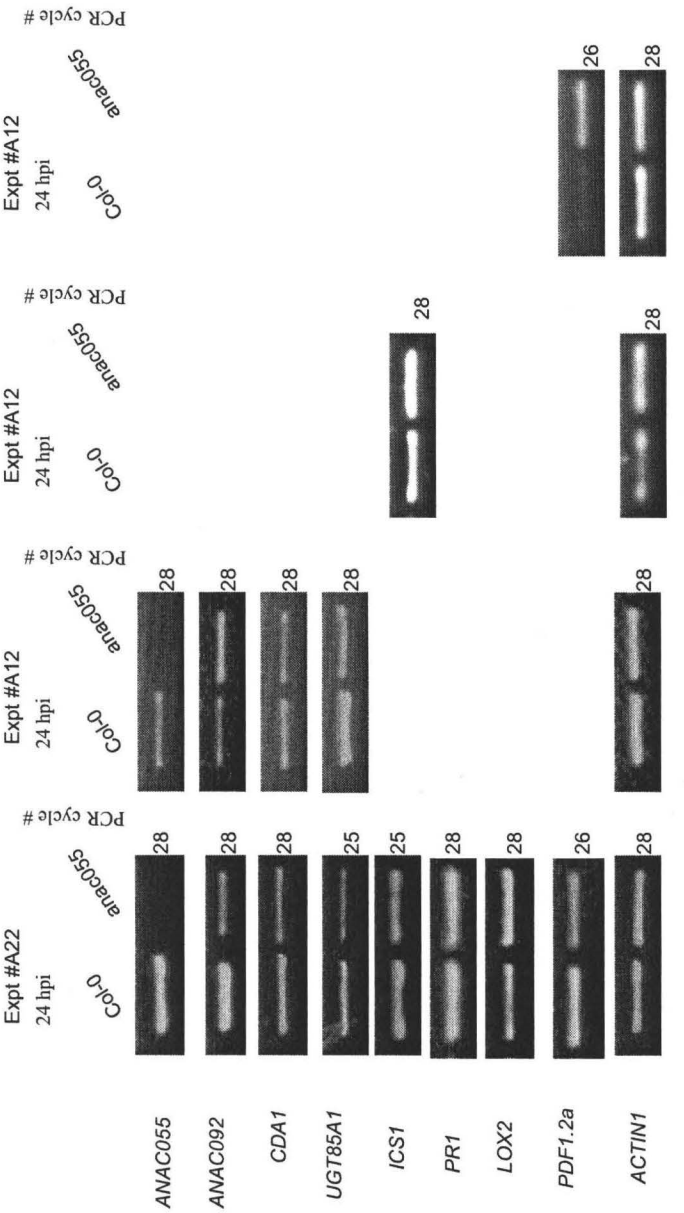

Expt #A22
24 hpi
Col-0
anac055
PCR cycle #
ANAC055
ANAC092
CDA1
UGT85A1
ICS1
PR1
LOX2
PDF1.2a
ACTIN1
28
25
26

Fig A29: RT-PCR analysis of *ANAC092*, *ANAC055*, *CDA1*, *UGT85A1*, *ICS1*, *PR1*, *LOX2*, and *PDF1.2a* expression in mature (6 wpg) Col-0 and *anac055* (ARR experiments A5, A47, and A50). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000.

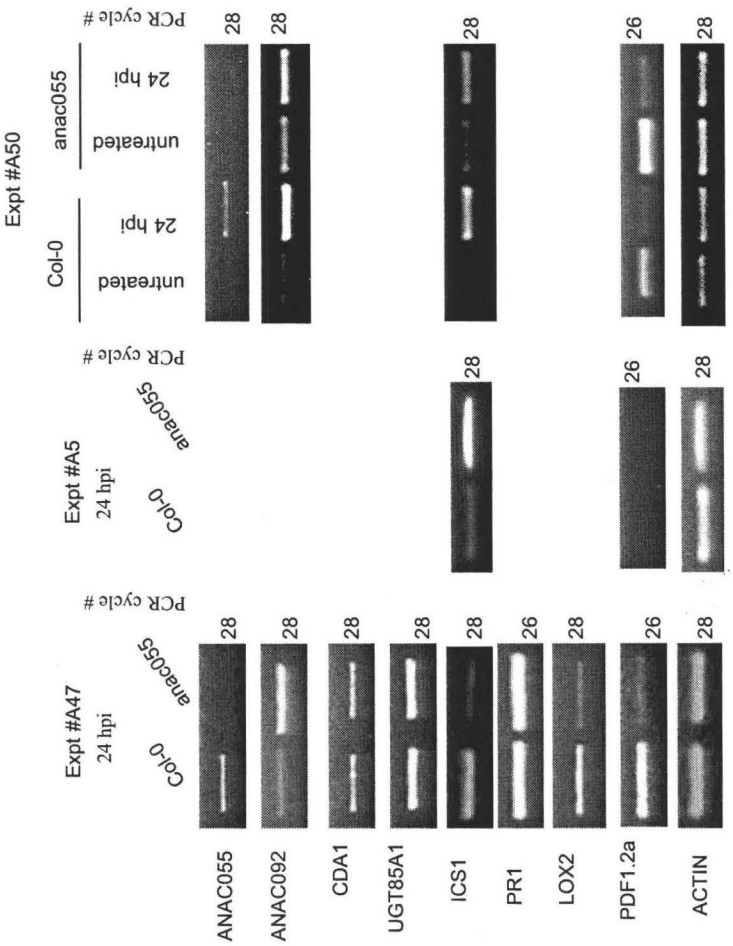

Expt #A50
Col-0
anac055
untreated
24 hpi
untreated
24 hpi
PCR cycle #
28
28
28
26
28
Expt #A5
24 hpi
Col-0
anac055
PCR cycle #
28
26
28
Expt #A47
24 hpi
Col-0
anac055
PCR cycle #
28
28
28
28
28
26
28
26
28
ANAC055
ANAC092
CDA1
UGT85A1
ICS1
PR1
LOX2
PDF1.2a
ACTIN

Fig A30: RT-PCR analysis of *ANAC092*, *ANAC055*, *CDA1*, and *UGT85A1* expression in mature (6 wpg) Col-0, *lox2*, *sid2*, *coi1-17*, and *ein2-1* (ARR experiments A12, A44, A46, A49, and A51). Leaf samples were collected from untreated plants, wounded leaves (needless syringe pressed down on leaf to damage tissue but not break through the leaf), mock-inoculated plants (10 mM $MgCl_2$), and plants inoculated with *Pst* DC3000 and *Pst* DC3118 (coronatine-deficient). All samples were collected at 24 hrs after treatment. *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000.

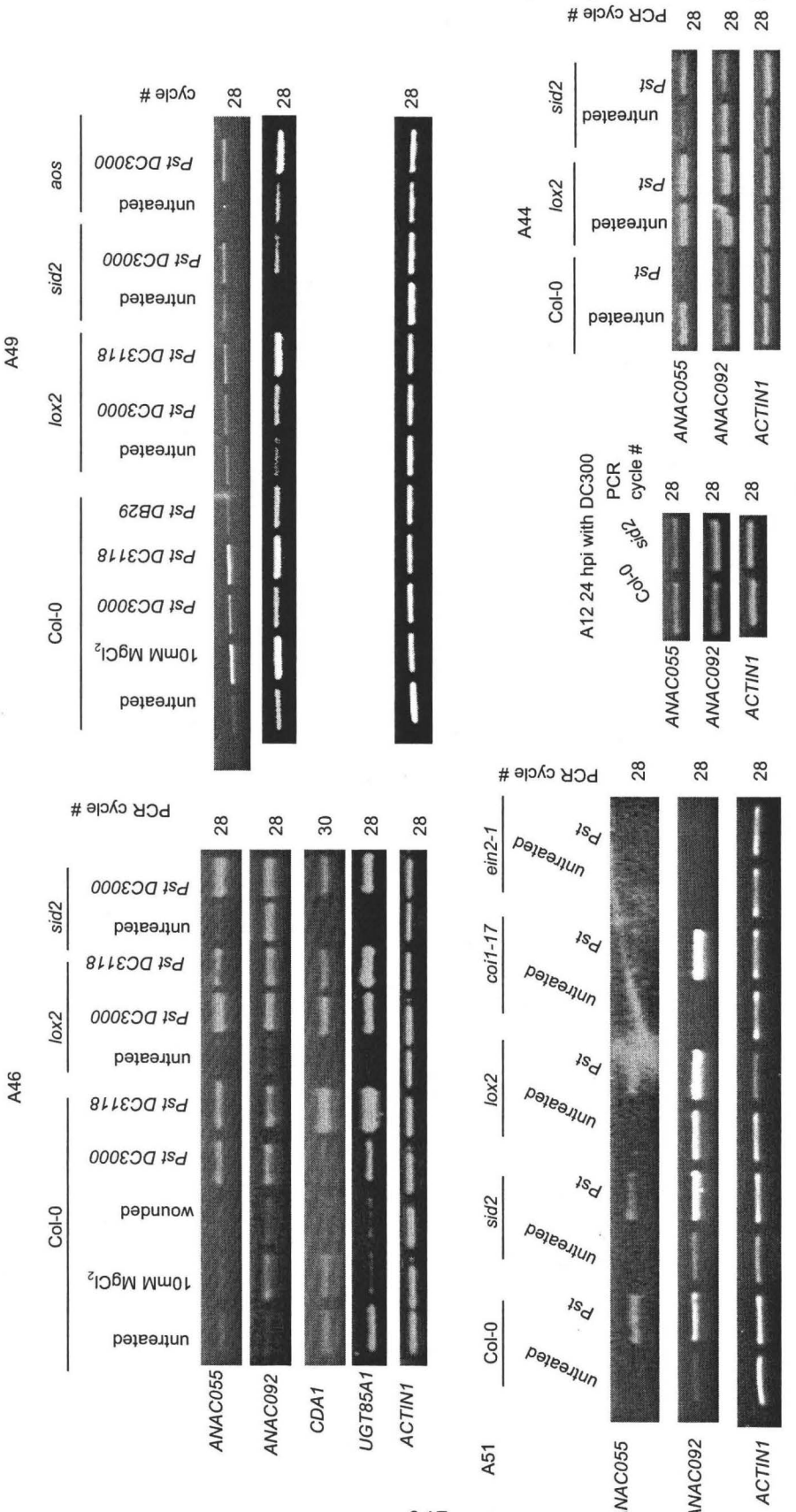
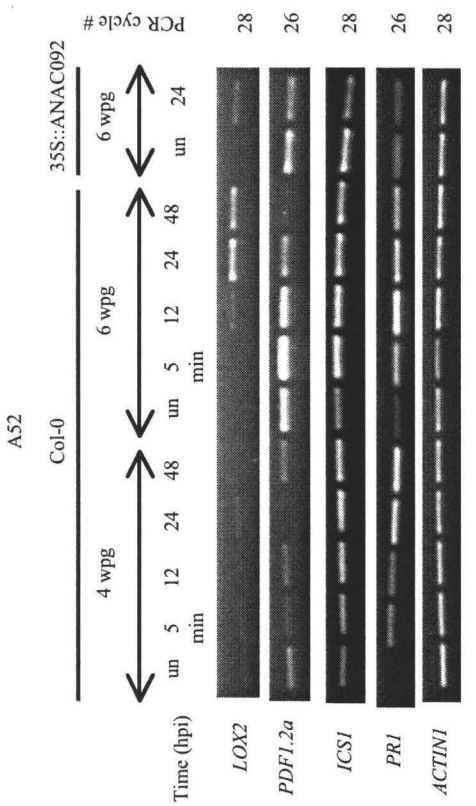

A46
Col-0
lox2
sid2
untreated
10mM MgCl2
wounded
Pst DC3000
Pst DC3118
untreated
Pst DC3000
Pst DC3118
untreated
Pst DC3000
PCR cycle #
28
28
30
28
28
ANAC055
ANAC092
CDA1
UGT85A1
ACTIN1
A49
Col-0
lox2
sid2
aos
untreated
10mM MgCl2
Pst DC3000
Pst DC3118
Pst DB29
untreated
Pst DC3000
Pst DC3118
untreated
Pst DC3000
untreated
Pst DC3000
cycle #
28
28
28
A12 24 hpi with DC300
Col-0
sid2
PCR cycle #
28
28
28
ANAC055
ANAC092
ACTIN1
A44
Col-0
lox2
sid2
untreated
Pst
untreated
Pst
untreated
Pst
PCR cycle #
28
28
28
ANAC055
ANAC092
ACTIN1
A51
Col-0
sid2
lox2
coi1-17
ein2-1
untreated
Pst
untreated
Pst
untreated
Pst
untreated
Pst
untreated
Pst
PCR cycle #
28
28
28
ANAC055
ANAC092
ACTIN1

Fig A31: RT-PCR analysis of *LOX2*, *PDF1.2a*, and *PR1* expression in ARR-incompetent Col-0 plants (4 wpg in A52 and 6 wpg in A56), Col-0 plant exhibiting ARR (6 wpg in A52 and 7 wpg in A56), and mature 35S:*ANAC092* (untreated and at 5 min, 12, 24, and 48 hpi). *ACTIN1* was used as a loading control. The number of PCR cycles used is shown. Plants were grown under short days (9 hr photoperiod) and inoculated with 10^6 cfu ml^{-1} virulent *Pst* DC3000. For details of *Pst* quantification refer to the legend of Fig 3.1.


A52
Col-0
35S::ANAC092
4 wpg
6 wpg
6 wpg
Time (hpi)
un 5 12 24 48 un 5 12 24 48 un 24
min
PCR cycle #
LOX2 28
PDF1.2a 26
ICS1 28
PR1 26
ACTIN1 28

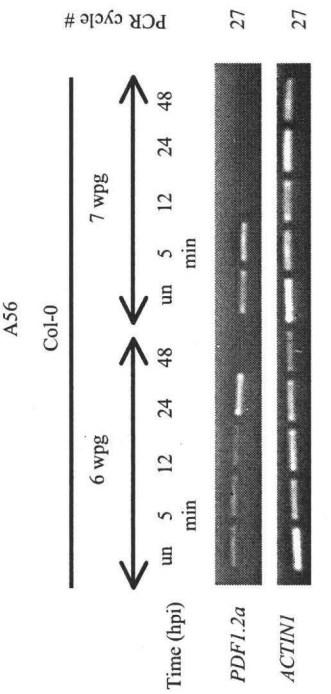
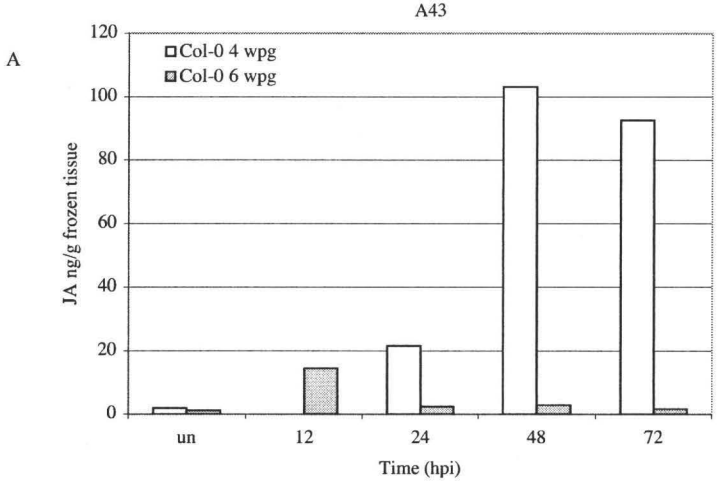
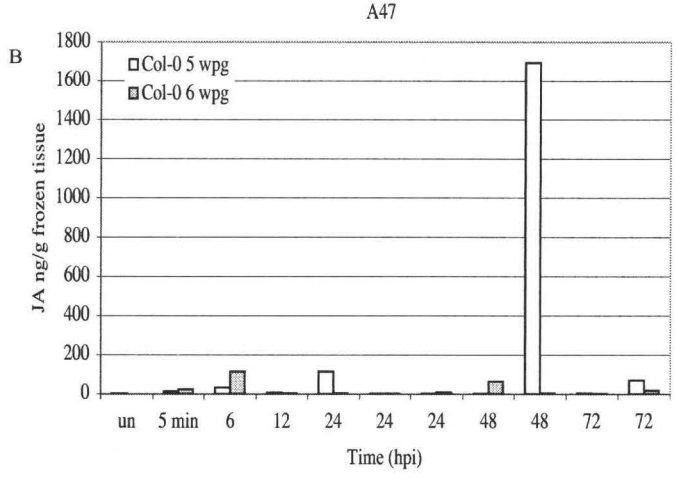
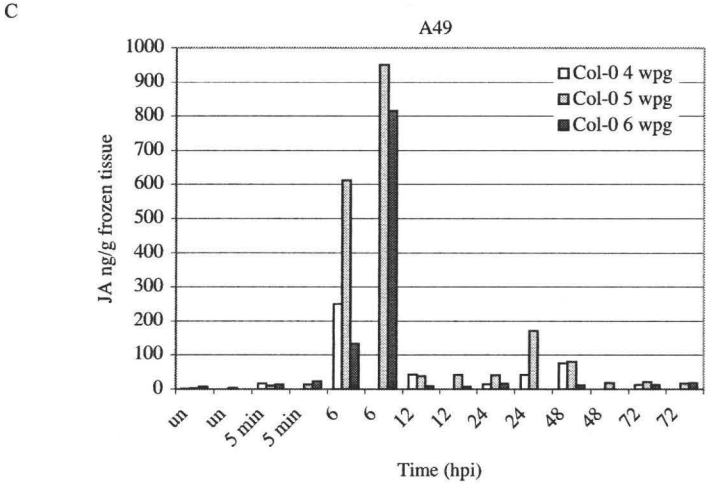

A56
Col-0
6 wpg
7 wpg
Time (hpi)
un 5 12 24 48 un 5 12 24 48
min
PCR cycle #
PDF1.2a 27
ACTIN1 27

Fig A32: JA levels in young (4 or 5 wpg) and mature (6 wpg) Col-0 (untreated to 72 hpi with 10^6 cfu ml^{-1} *Pst*). Samples were collected from ARR experiment A) A43, B) A47, C) A49, and D) A52.


A
A43
Col-0 4 wpg
Col-0 6 wpg
JA ng/g frozen tissue
Time (hpi)
un
12
24
48
72


B
A47
Col-0 5 wpg
Col-0 6 wpg
JA ng/g frozen tissue
Time (hpi)
un
5 min
6
12
24
24
24
48
48
72
72


C
A49
Col-0 4 wpg
Col-0 5 wpg
Col-0 6 wpg
JA ng/g frozen tissue
Time (hpi)

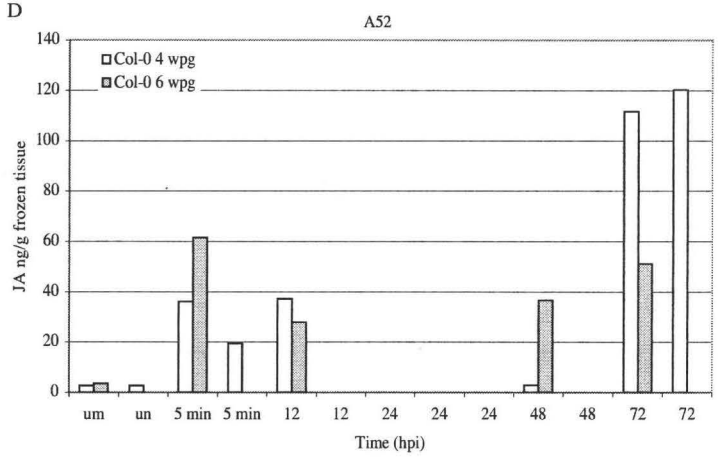
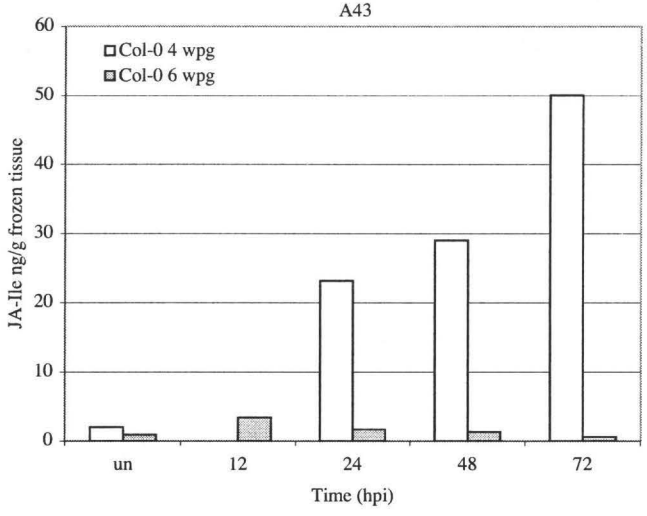
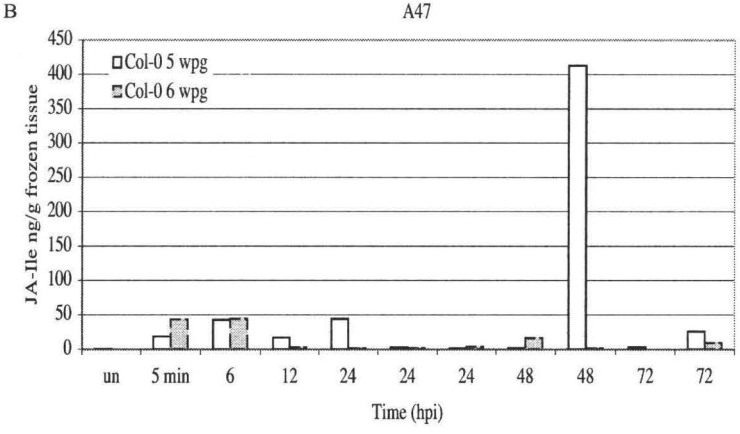

D
A52
Col-0 4 wpg
Col-0 6 wpg
JA ng/g frozen tissue
Time (hpi)
um
un
5 min
5 min
12
12
24
24
24
48
48
72
72

Fig A33: JA-Ile levels in young (4 or 5 wpg) and mature (6 wpg) Col-0 (untreated to 72 hpi with 10^6 cfu ml^{-1} *Pst*). Samples were collected from ARR experiment A) A43, B) A47, C) A49, and D) A52.

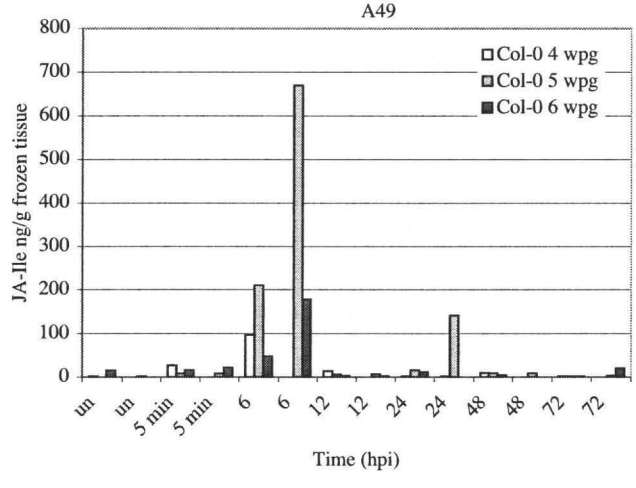
A


A43
Col-0 4 wpg
Col-0 6 wpg
JA-Ile ng/g frozen tissue
0
10
20
30
40
50
60
un
12
24
48
72
Time (hpi)

B


A47
Col-0 5 wpg
Col-0 6 wpg
JA-Ile ng/g frozen tissue
0
50
100
150
200
250
300
350
400
450
un
5 min
6
12
24
24
24
48
48
72
72
Time (hpi)

C


A49
Col-0 4 wpg
Col-0 5 wpg
Col-0 6 wpg
JA-Ile ng/g frozen tissue
0
100
200
300
400
500
600
700
800
un
un
5 min
5 min
6
6
12
12
24
24
48
48
72
72
Time (hpi)

D

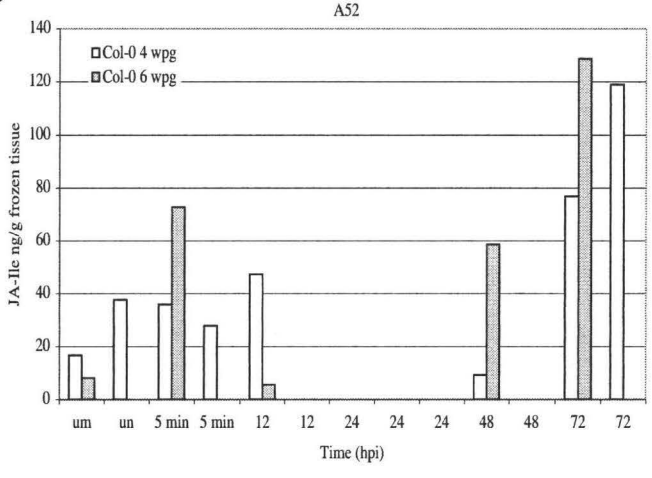

A52
Col-0 4 wpg
Col-0 6 wpg
JA-Ile ng/g frozen tissue
0
20
40
60
80
100
120
140
um
un
5 min
5 min
12
12
24
24
24
48
48
72
72
Time (hpi)

Fig A34: OPDA levels in young (4 or 5 wpg) and mature (6 wpg) Col-0 (untreated to 72 hpi with 10^6 cfu ml^{-1} *Pst*). Samples were collected from ARR experiment A) A43, B) A47, C) A49, and D) A52.

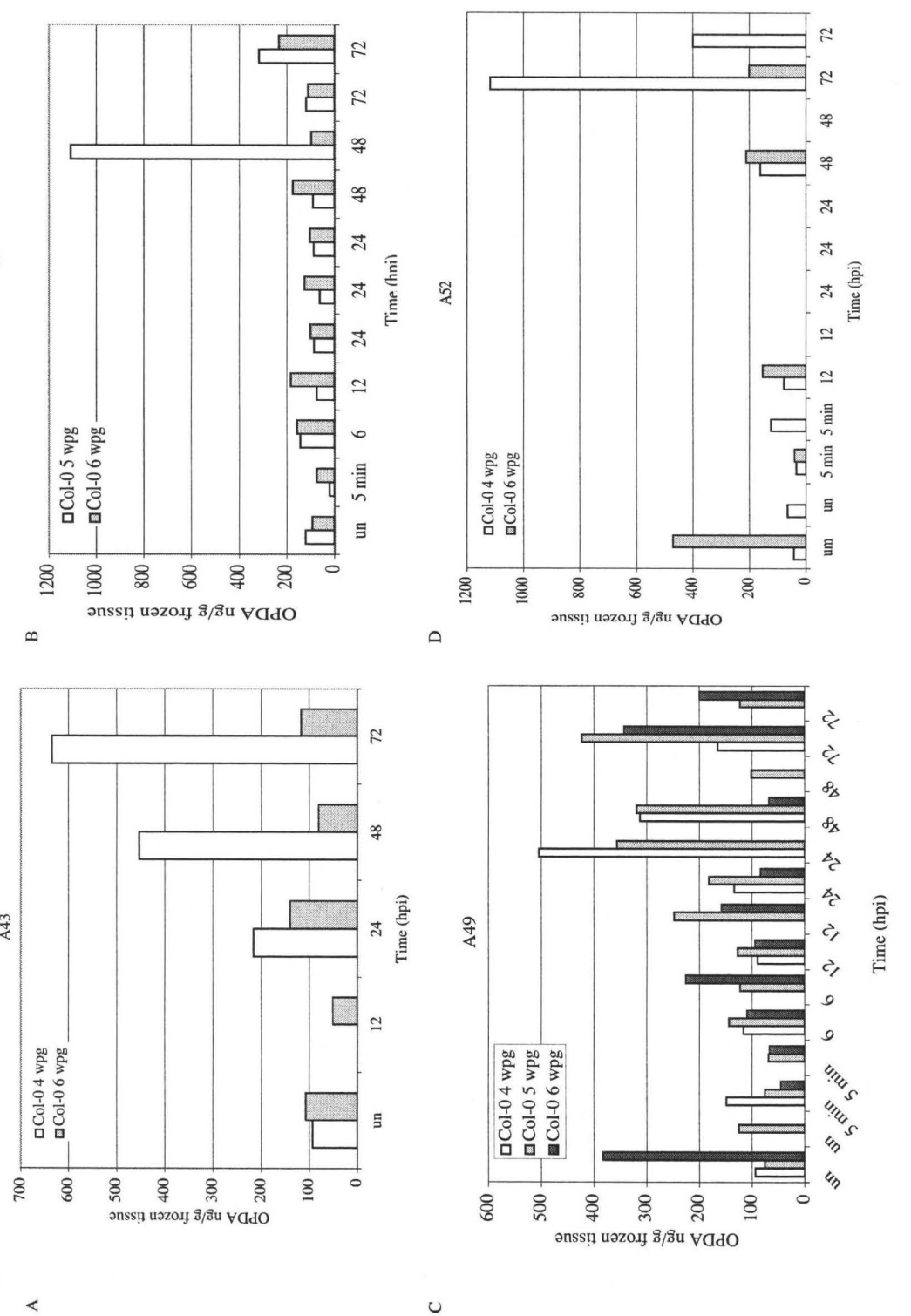

A
A43
Col-0 4 wpg
Col-0 6 wpg
OPDA ng/g frozen tissue
Time (hpi)
B
Col-0 5 wpg
Col-0 6 wpg
OPDA ng/g frozen tissue
Time (hpi)
C
A49
Col-0 4 wpg
Col-0 5 wpg
Col-0 6 wpg
OPDA ng/g frozen tissue
Time (hpi)
D
A52
Col-0 4 wpg
Col-0 6 wpg
OPDA ng/g frozen tissue
Time (hpi)

Fig A35: Free SA levels in young (4 or 5 wpg) and mature (6 wpg) Col-0 (untreated to 72 hpi with 10^6 cfu ml^{-1} *Pst*). Samples were collected from ARR experiment A) A43, B) A47, C) A49, and D) A52.

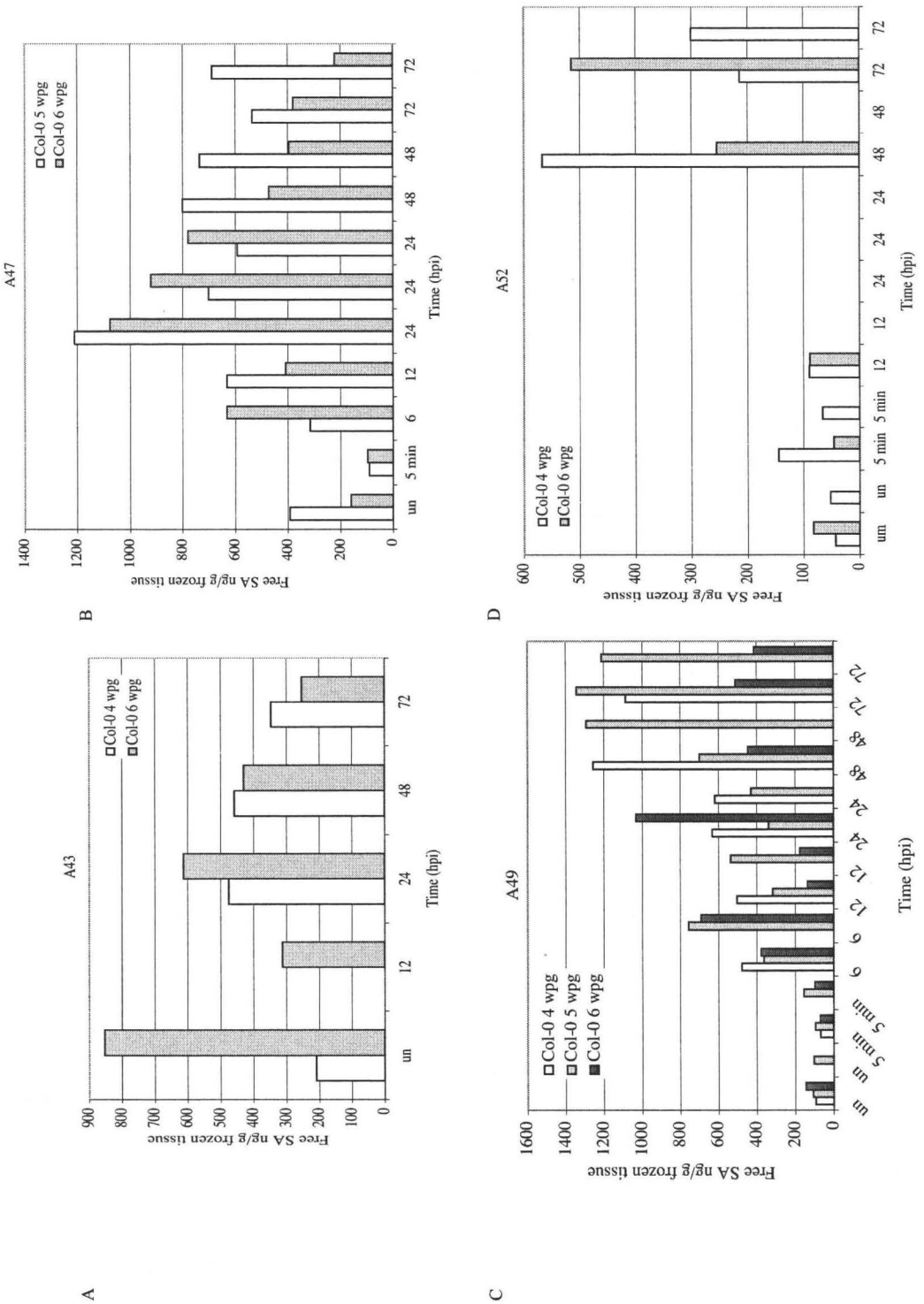

A
A43
Col-0 4 wpg
Col-0 6 wpg
Free SA ng/g frozen tissue
Time (hpi)
B
A47
Col-0 5 wpg
Col-0 6 wpg
Free SA ng/g frozen tissue
Time (hpi)
C
A49
Col-0 4 wpg
Col-0 5 wpg
Col-0 6 wpg
Free SA ng/g frozen tissue
Time (hpi)
D
A52
Col-0 4 wpg
Col-0 6 wpg
Free SA ng/g frozen tissue
Time (hpi)

Fig A36: IAA levels in young (4 or 5 wpg) and mature (6 wpg) Col-0 (untreated to 72 hpi with 10^6 cfu ml^{-1} *Pst*). Samples were collected from ARR experiment A) A43, B) A47, C) A49, and D) A52.

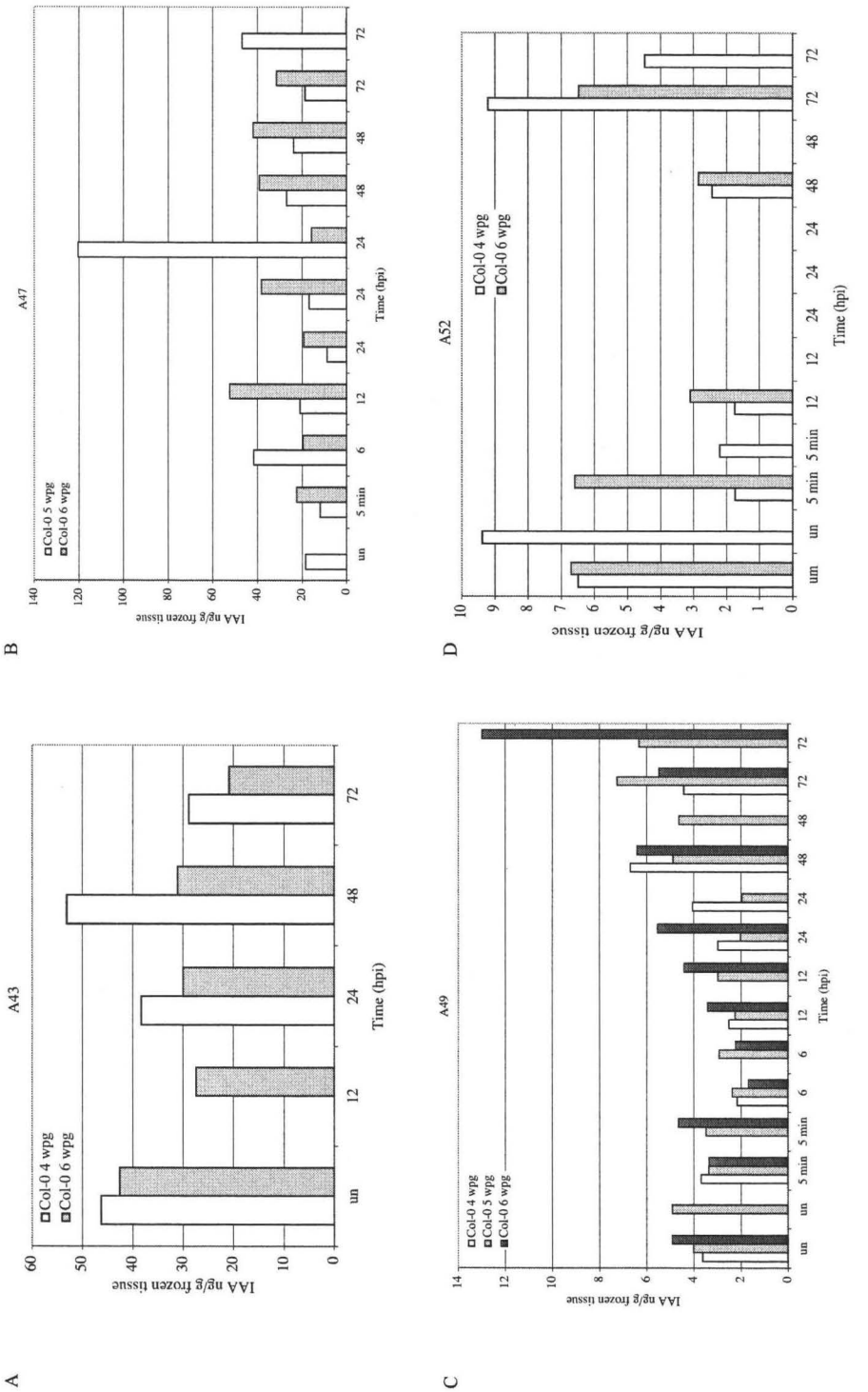
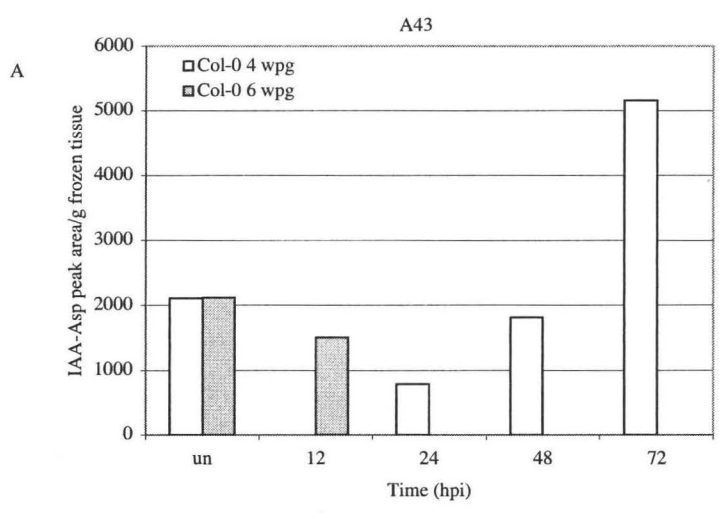
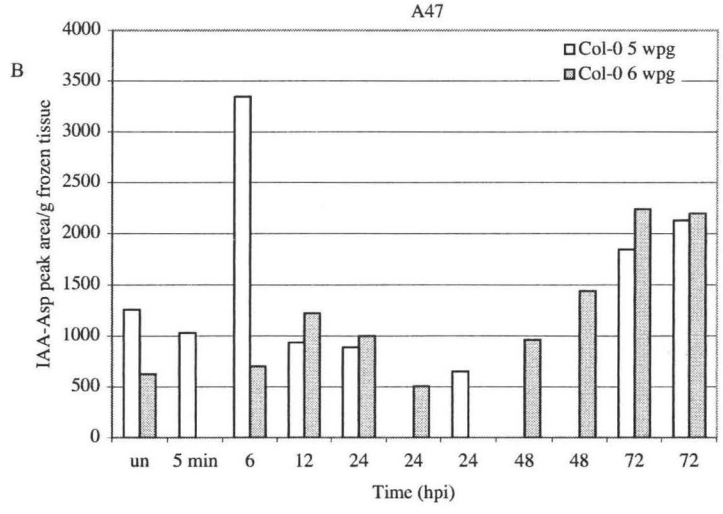
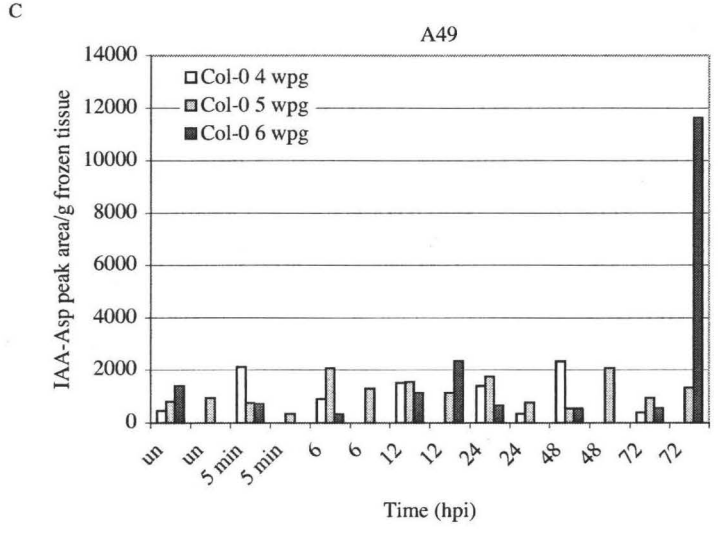

A
A43
Col-0 4 wpg
Col-0 6 wpg
IAA ng/g frozen tissue
Time (hpi)
un
12
24
48
72
B
A47
Col-0 5 wpg
Col-0 6 wpg
IAA ng/g frozen tissue
Time (hpi)
un
5 min
6
12
24
48
72
C
A49
Col-0 4 wpg
Col-0 5 wpg
Col-0 6 wpg
IAA ng/g frozen tissue
Time (hpi)
un
5 min
6
12
24
48
72
D
A52
Col-0 4 wpg
Col-0 6 wpg
IAA ng/g frozen tissue
Time (hpi)
um
un
5 min
12
24
48
72

Fig A37: IAA-Asp levels in young (4 or 5 wpg) and mature (6 wpg) Col-0 (untreated to 72 hpi with 10^6 cfu ml^{-1} *Pst*). Samples were collected from ARR experiment A) A43, B) A47, C) A49, and D) A52.


A
A43
Col-0 4 wpg
Col-0 6 wpg
IAA-Asp peak area/g frozen tissue
6000
5000
4000
3000
2000
1000
0
un
12
24
48
72
Time (hpi)


B
A47
Col-0 5 wpg
Col-0 6 wpg
IAA-Asp peak area/g frozen tissue
4000
3500
3000
2500
2000
1500
1000
500
0
un
5 min
6
12
24
24
24
48
48
72
72
Time (hpi)


C
A49
Col-0 4 wpg
Col-0 5 wpg
Col-0 6 wpg
IAA-Asp peak area/g frozen tissue
14000
12000
10000
8000
6000
4000
2000
0
un
un
5 min
5 min
6
6
12
12
24
24
48
48
72
72
Time (hpi)

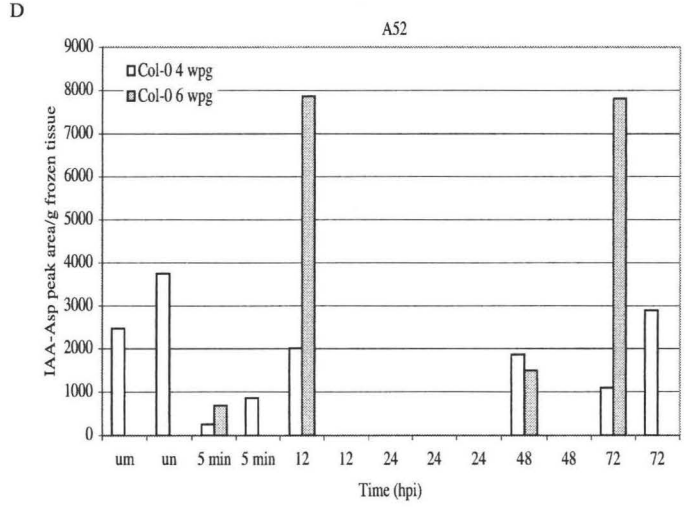
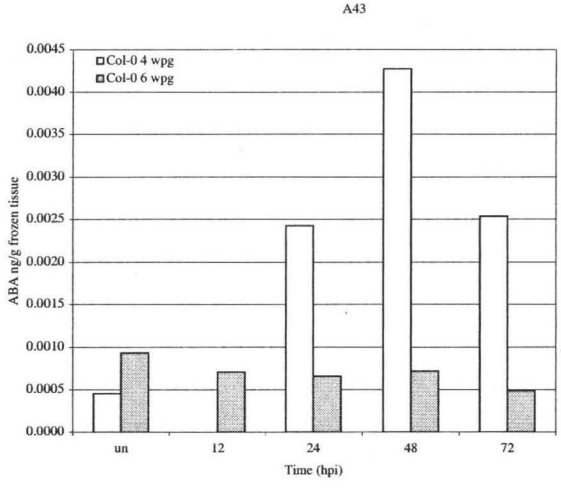
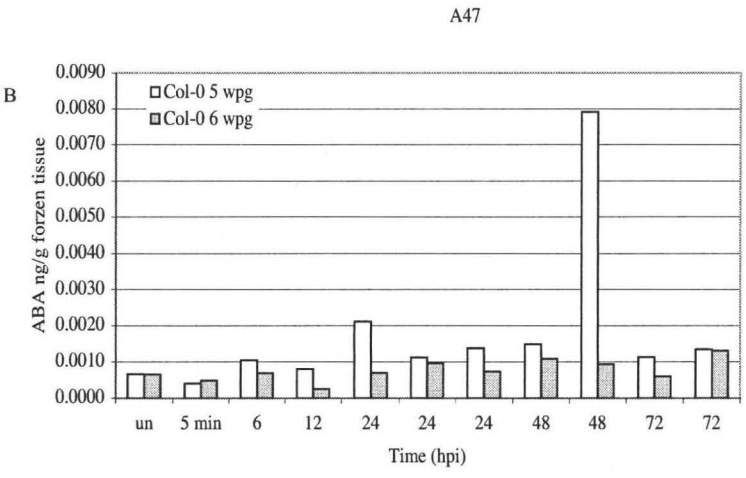

D
A52
Col-0 4 wpg
Col-0 6 wpg
IAA-Asp peak area/g frozen tissue
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
um
un
5 min
5 min
12
12
24
24
24
48
48
72
72
Time (hpi)

Fig A38: ABA levels in young (4 or 5 wpg) and mature (6 wpg) Col-0 (untreated to 72 hpi with 10^6 cfu ml^{-1} *Pst*). Samples were collected from ARR experiment A) A43, B) A47, C) A49, and D) A52.

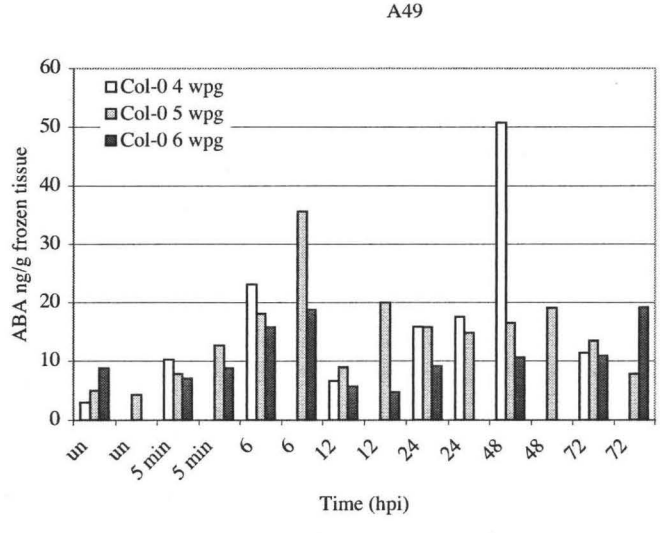
A


A43
Col-0 4 wpg
Col-0 6 wpg
ABA ng/g frozen tissue
0.0045
0.0040
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
un
12
24
48
72
Time (hpi)

B


A47
Col-0 5 wpg
Col-0 6 wpg
ABA ng/g forzen tissue
0.0090
0.0080
0.0070
0.0060
0.0050
0.0040
0.0030
0.0020
0.0010
0.0000
un
5 min
6
12
24
24
24
48
48
72
72
Time (hpi)

C


A49
Col-0 4 wpg
Col-0 5 wpg
Col-0 6 wpg
ABA ng/g frozen tissue
60
50
40
30
20
10
0
un
un
5 min
5 min
6
6
12
12
24
24
48
48
72
72
Time (hpi)

D

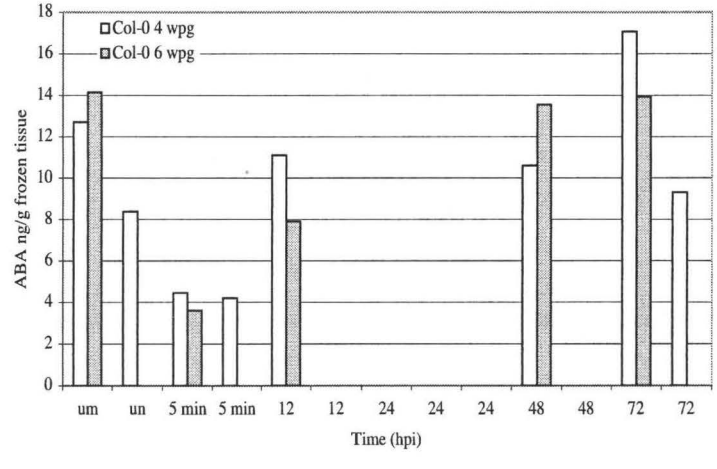
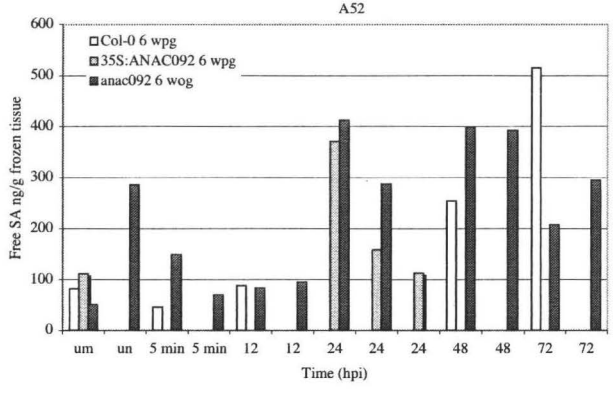
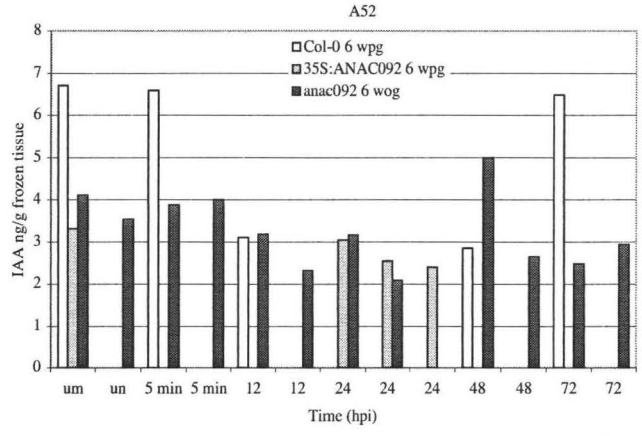

A52
Col-0 4 wpg
Col-0 6 wpg
ABA ng/g frozen tissue
18
16
14
12
10
8
6
4
2
0
um
un
5 min
5 min
12
12
24
24
24
48
48
72
72
Time (hpi)

Fig A39: A) Free SA, B) IAA, C) IAA-Asp, and D) ABA levels in mature (6 wpg) Col-0, 35S:*ANAC092*, and *anac092* (untreated to 72 hpi with 10^6 cfu ml^{-1} *Pst*). Samples were collected from ARR experiment A52.

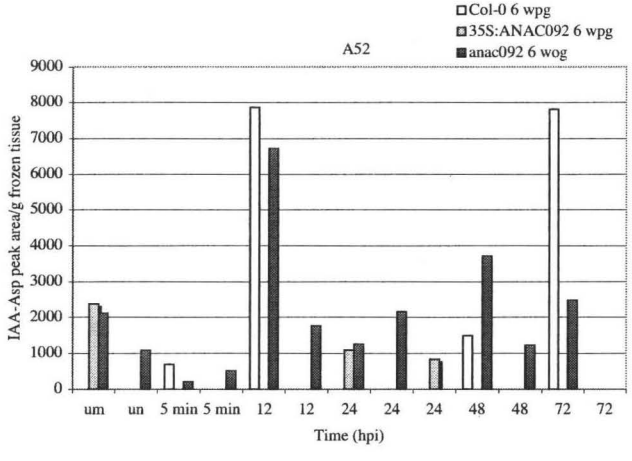
A


A52
Col-0 6 wpg
35S:ANAC092 6 wpg
anac092 6 wog
Free SA ng/g frozen tissue
600
500
400
300
200
100
0
um un 5 min 5 min 12 12 24 24 24 48 48 72 72
Time (hpi)

B


A52
Col-0 6 wpg
35S:ANAC092 6 wpg
anac092 6 wog
IAA ng/g frozen tissue
8
7
6
5
4
3
2
1
0
um un 5 min 5 min 12 12 24 24 24 48 48 72 72
Time (hpi)

C


Col-0 6 wpg
35S:ANAC092 6 wpg
anac092 6 wog
A52
IAA-Asp peak area/g frozen tissue
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
um un 5 min 5 min 12 12 24 24 24 48 48 72 72
Time (hpi)

D

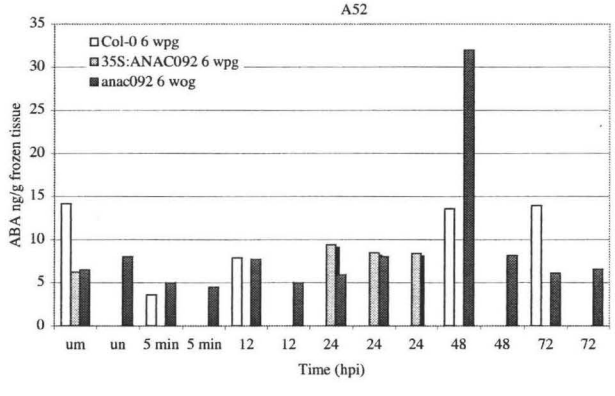

A52
Col-0 6 wpg
35S:ANAC092 6 wpg
anac092 6 wog
ABA ng/g frozen tissue
35
30
25
20
15
10
5
0
um un 5 min 5 min 12 12 24 24 24 48 48 72 72
Time (hpi)

Fig A40: A) JA, B) OPDA, and C) JA-Ile levels in mature (6 wpg) Col-0, 35S:*ANAC092*, and *anac092* (untreated to 72 hpi with 10^6 cfu ml^{-1} *Pst*). Samples were collected from ARR experiment A52.

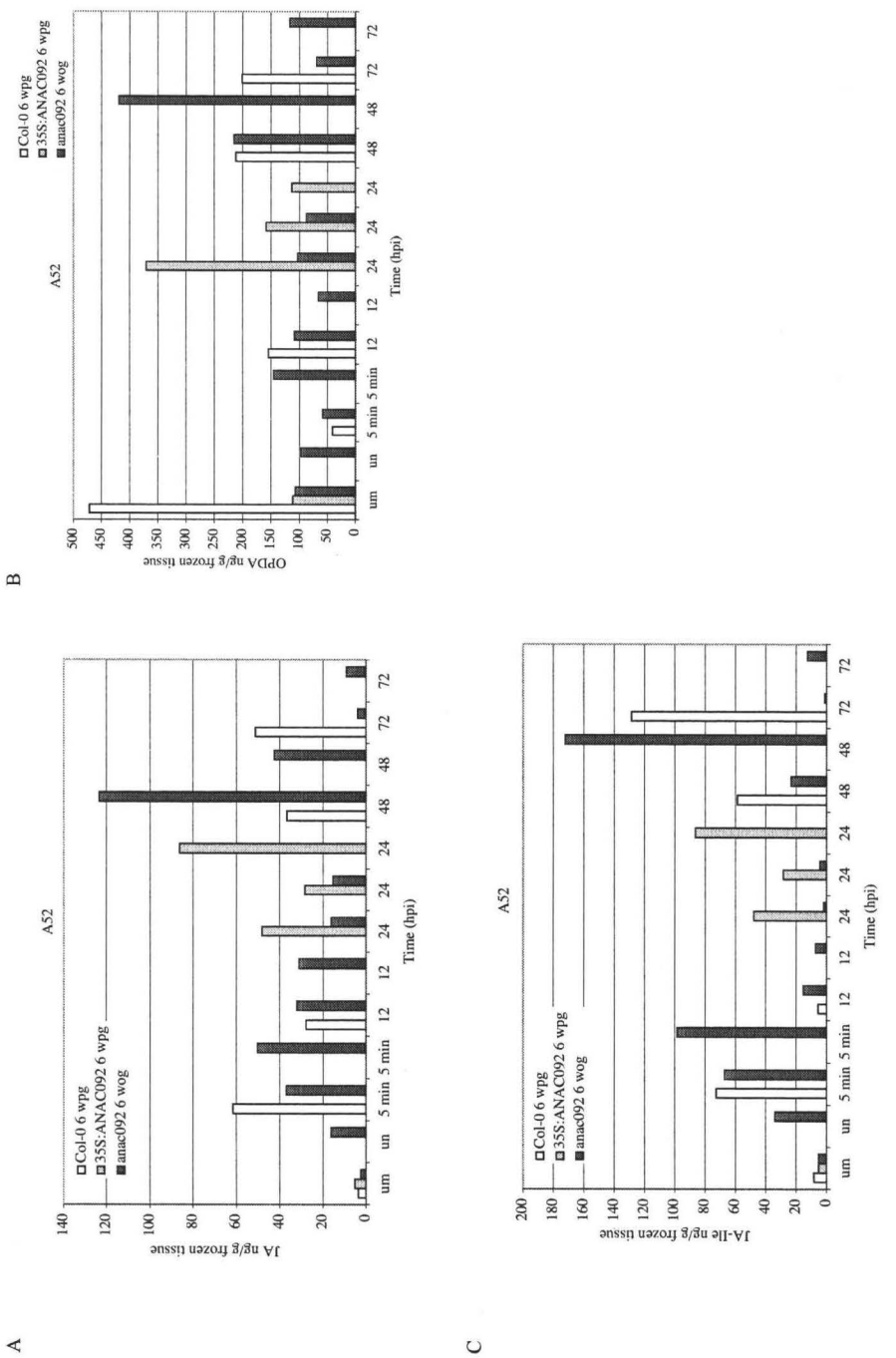

A
A52
Col-0 6 wpg
35S:ANAC092 6 wpg
anac092 6 wog
JA ng/g frozen tissue
Time (hpi)
um un 5 min 5 min 12 12 24 24 24 48 48 72 72
0 20 40 60 80 100 120 140
B
A52
Col-0 6 wpg
35S:ANAC092 6 wpg
anac092 6 wog
OPDA ng/g frozen tissue
Time (hpi)
um un 5 min 5 min 12 12 24 24 24 48 48 72 72
0 50 100 150 200 250 300 350 400 450 500
C
A52
Col-0 6 wpg
35S:ANAC092 6 wpg
anac092 6 wog
JA-Ile ng/g frozen tissue
Time (hpi)
um un 5 min 5 min 12 12 24 24 24 48 48 72 72
0 20 40 60 80 100 120 140 160 180 200

Fig A41: A) Free SA, and B) OPDA levels in young (4 and 5 wpg) and mature (6 wpg) Col-0, mature *lox2*, and mature *sid2* (untreated to 72 hpi with 10^6 cfu ml^{-1} *Pst*). Mock-inoculated (10 mM $MgCl_2$) samples from ARR-incompetent plants (5 wpg) and plants exhibiting ARR mature (6 wpg) Col-0 are included. Samples were collected from ARR experiment A49.

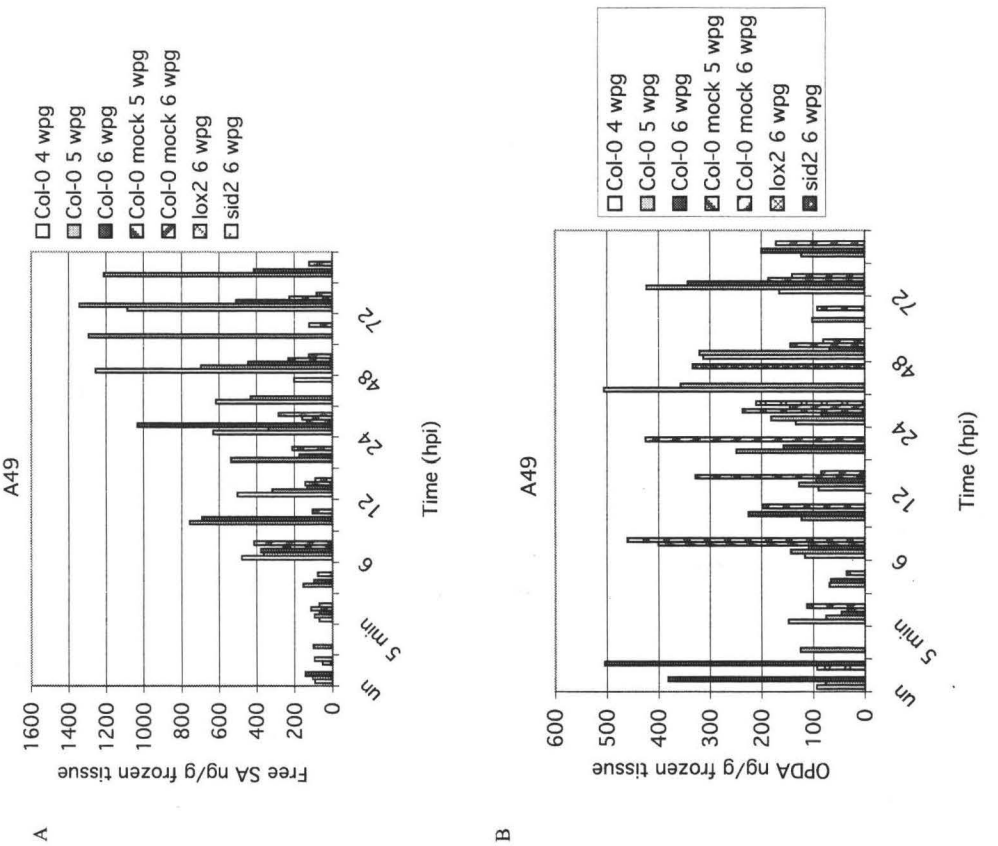

A
A49
Free SA ng/g frozen tissue
1600
1400
1200
1000
800
600
400
200
0
Un
5 min
6
12
24
48
72
Time (hpi)
Col-0 4 wpg
Col-0 5 wpg
Col-0 6 wpg
Col-0 mock 5 wpg
Col-0 mock 6 wpg
lox2 6 wpg
sid2 6 wpg
B
A49
OPDA ng/g frozen tissue
600
500
400
300
200
100
0
Un
5 min
6
12
24
48
72
Time (hpi)
Col-0 4 wpg
Col-0 5 wpg
Col-0 6 wpg
Col-0 mock 5 wpg
Col-0 mock 6 wpg
lox2 6 wpg
sid2 6 wpg

Fig A42: A) IAA-Asp, B) IAA-Asp (without 72 hpi sample from six week-old Col-0), C) ABA, and D) IAA levels in young (4 and 5 wpg) and mature (6 wpg) Col-0, mature *lox2*, and mature *sid2* (untreated to 72 hpi with 10^6 cfu ml^{-1} *Pst*). Mock-inoculated (10 mM $MgCl_2$) samples from ARR-incompetent plants (5 wpg) and plants exhibiting ARR (6 wpg) are included. Samples were collected from ARR experiment A49.

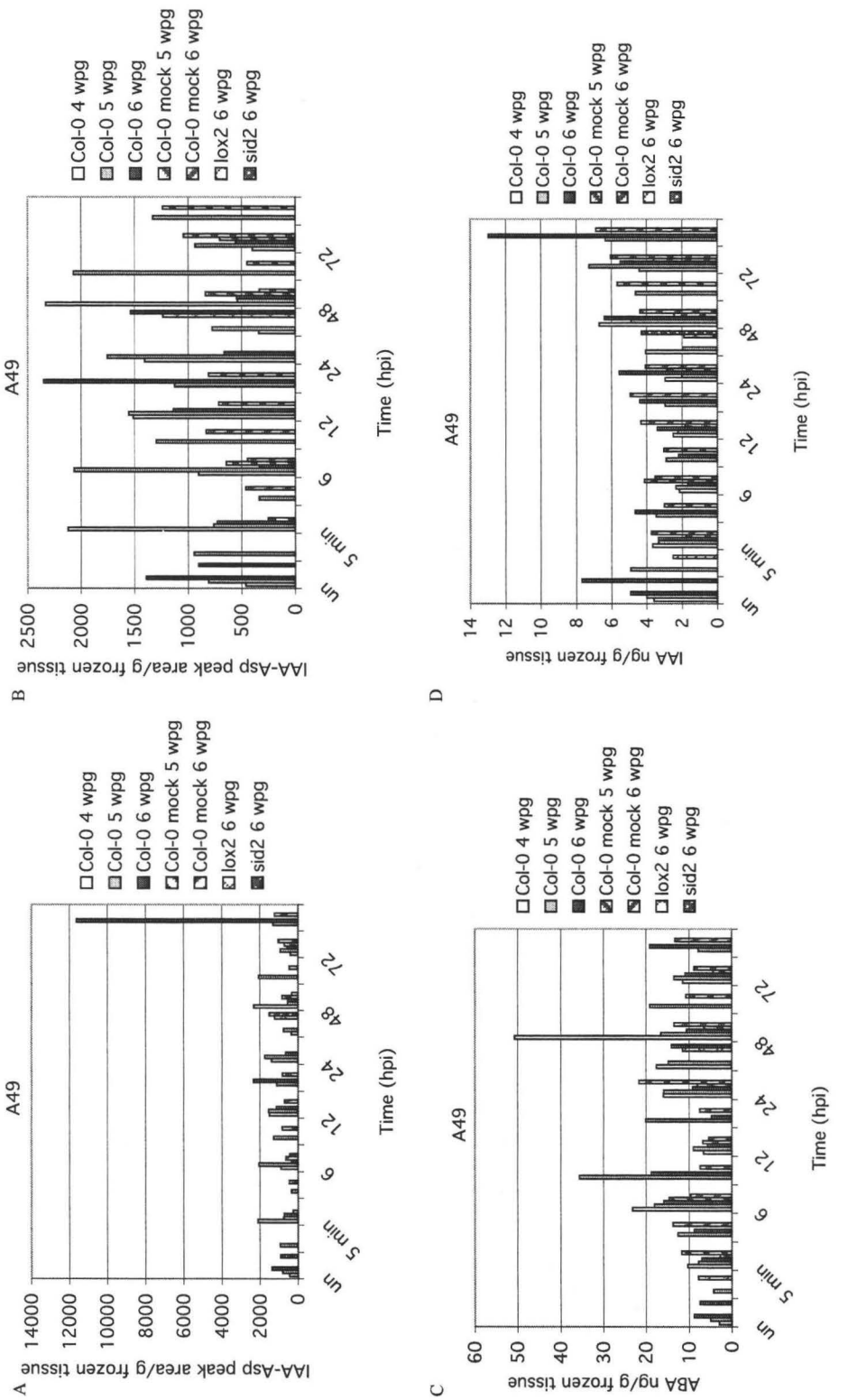

A
IAA-Asp peak area/g frozen tissue
14000
12000
10000
8000
6000
4000
2000
0
A49
Col-0 4 wpg
Col-0 5 wpg
Col-0 6 wpg
Col-0 mock 5 wpg
Col-0 mock 6 wpg
lox2 6 wpg
sid2 6 wpg
un
5 min
6
12
24
48
72
Time (hpi)
B
IAA-Asp peak area/g frozen tissue
2500
2000
1500
1000
500
0
C
ABA ng/g frozen tissue
60
50
40
30
20
10
0
D
IAA ng/g frozen tissue
14
12
10
8
6
4
2
0

Fig A43: A) JA-Ile, B) JA-Ile (without 12 hpi sample from five week-old Col-0), C) JA, and D) JA (without 6 and 12 hpi sample from five week-old Col-0 and without 12 hpi sample from six week-old Col-0) levels in young (4 and 5 wpg) and mature (6 wpg) Col-0, mature *lox2*, and mature *sid2* (untreated to 72 hpi with 10^6 cfu ml^{-1} *Pst*). Mock-inoculated (10 mM $MgCl_2$) samples from ARR-incompetent plants (5 wpg) and plants exhibiting ARR (6 wpg) are included. Samples were collected from ARR experiment A49.

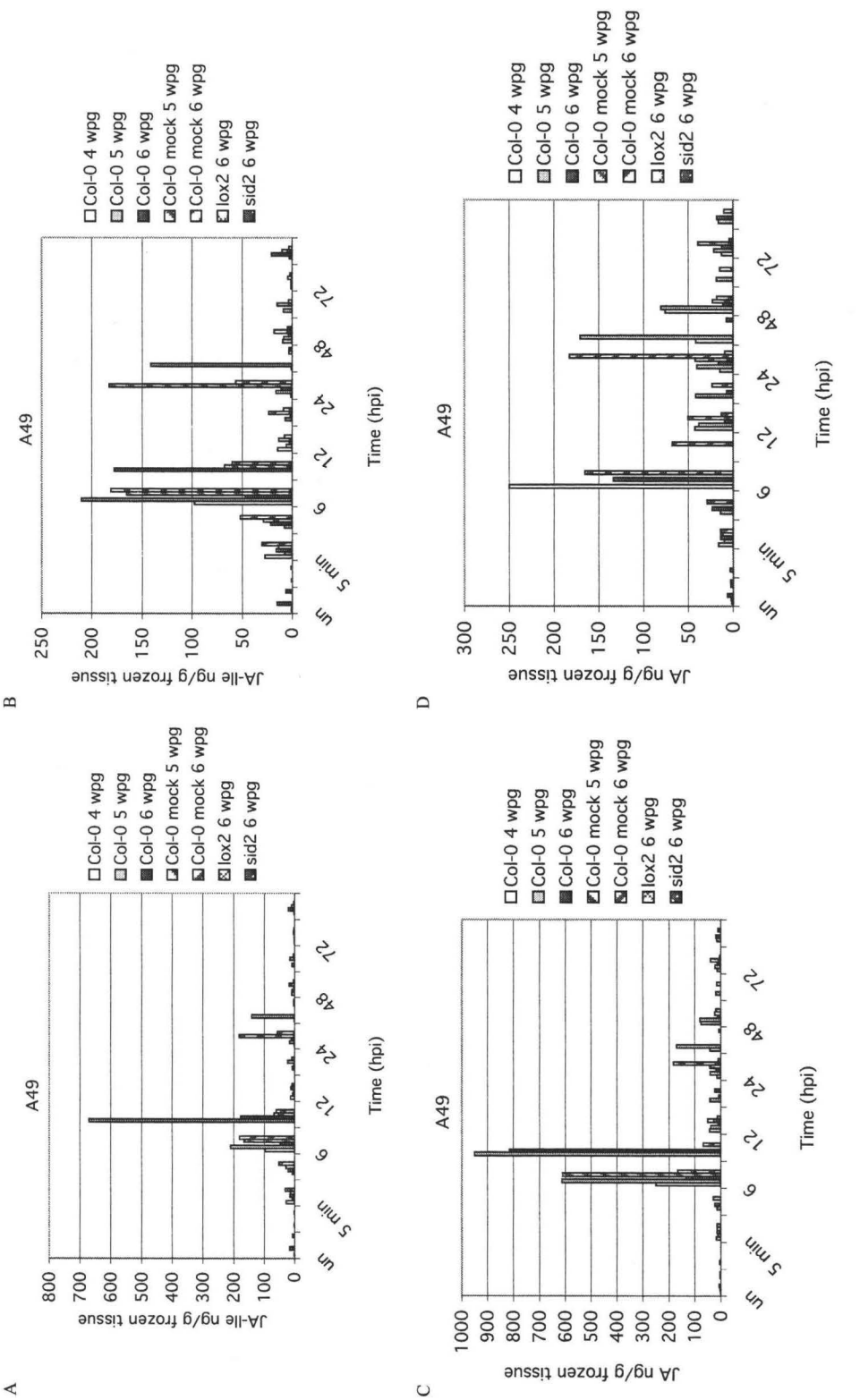
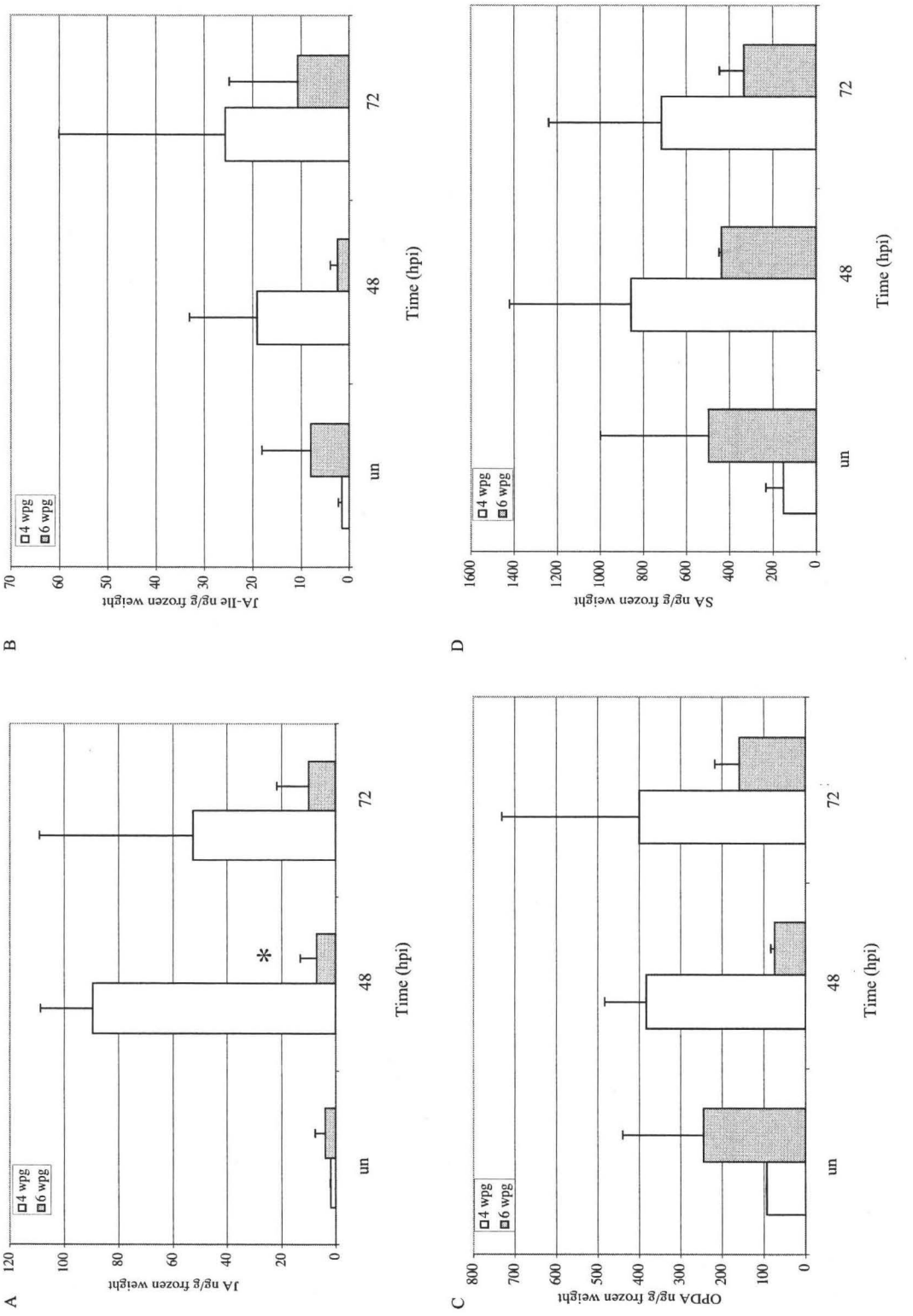
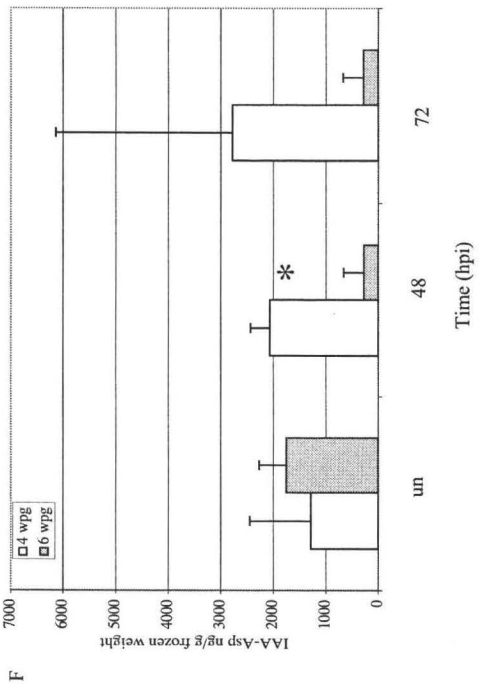
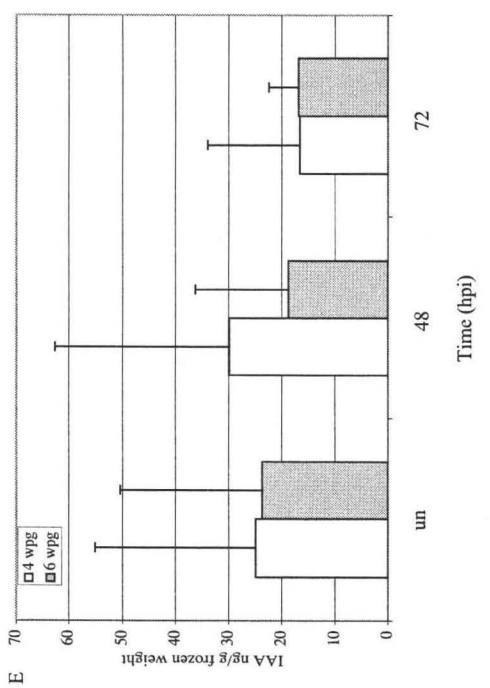

A
A49
JA-Ile ng/g frozen tissue
Time (hpi)
Col-0 4 wpg
Col-0 5 wpg
Col-0 6 wpg
Col-0 mock 5 wpg
Col-0 mock 6 wpg
lox2 6 wpg
sid2 6 wpg
B
A49
JA-Ile ng/g frozen tissue
Time (hpi)
C
A49
JA ng/g frozen tissue
Time (hpi)
D
A49
JA ng/g frozen tissue
Time (hpi)

Fig A44: Average (n=2 +/- SD) levels of A) JA, B) JA-Ile, C) OPDA, D) free SA, E) IAA, F) IAA-Asp, and G) ABA in young (4 wpg) and mature (6 wpg) plants inoculated with 10^6 cfu ml^{-1} *Pst* in experiments A43 and A49. * $P < 0.05$ Student's T-test.


A
JA ng/g frozen weight
4 wpg
6 wpg
un
48
72
Time (hpi)
*
B
JA-Ile ng/g frozen weight
4 wpg
6 wpg
un
48
72
Time (hpi)
C
OPDA ng/g frozen weight
4 wpg
6 wpg
un
48
72
Time (hpi)
D
SA ng/g frozen weight
4 wpg
6 wpg
un
48
72
Time (hpi)


F
IAA-Asp ng/g frozen weight
7000
6000
5000
4000
3000
2000
1000
0
4 wpg
6 wpg
*
un
48
72
Time (hpi)


E
IAA ng/g frozen weight
70
60
50
40
30
20
10
0
4 wpg
6 wpg
un
48
72
Time (hpi)

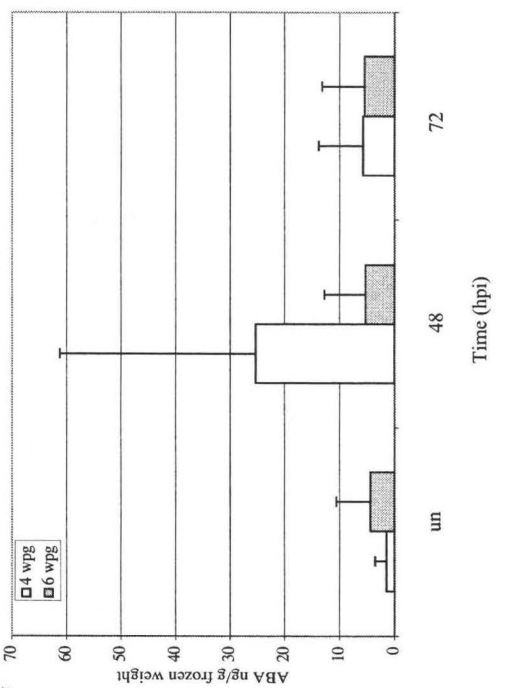
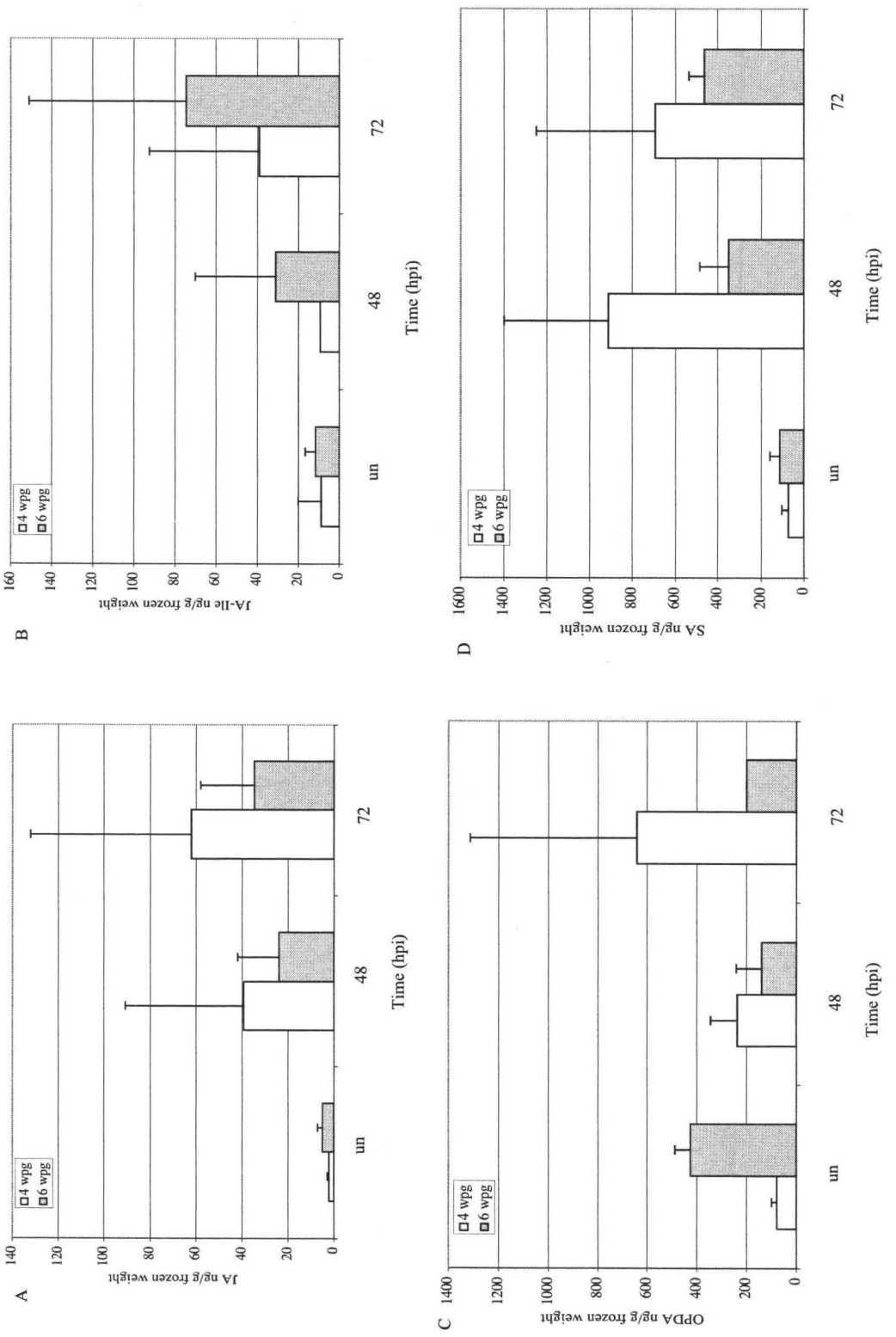
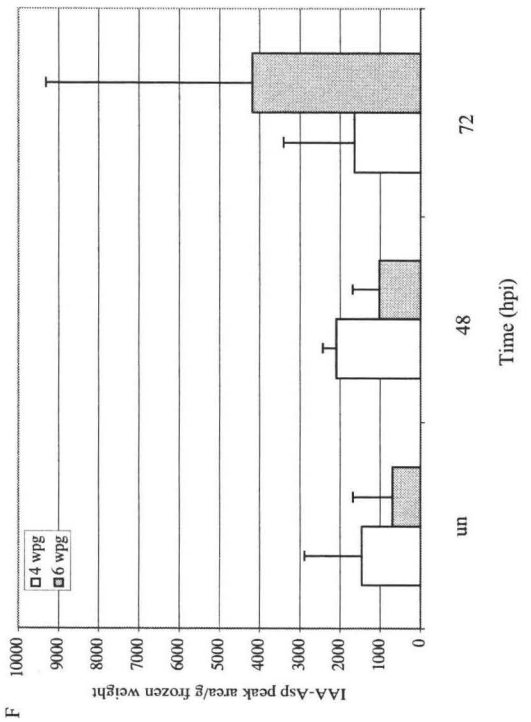
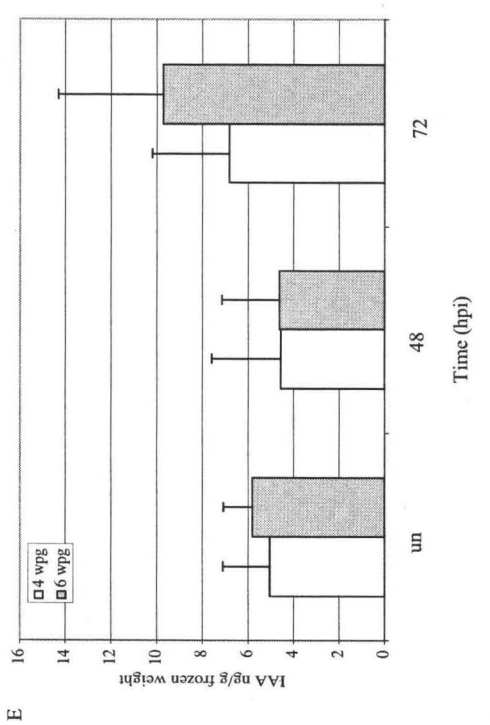

G
ABA ng/g frozen weight
70
60
50
40
30
20
10
0
4 wpg
6 wpg
un
48
72
Time (hpi)

Fig A45: Average (n=2 +/- SD) levels of A) JA, B) JA-Ile, C) OPDA, D) free SA, E) IAA, F) IAA-Asp, and G) ABA in young (4 wpg) and mature (6 wpg) plants inoculated with 10^6 cfu ml^{-1} *Pst* in experiments A49 and A52.


A
JA ng/g frozen weight
0
20
40
60
80
100
120
140
4 wpg
6 wpg
un
48
72
Time (hpi)
B
JA-Ile ng/g frozen weight
0
20
40
60
80
100
120
140
160
4 wpg
6 wpg
un
48
72
Time (hpi)
C
OPDA ng/g frozen weight
0
200
400
600
800
1000
1200
1400
4 wpg
6 wpg
un
48
72
Time (hpi)
D
SA ng/g frozen weight
0
200
400
600
800
1000
1200
1400
1600
4 wpg
6 wpg
un
48
72
Time (hpi)


F
IAA-Asp peak area/g frozen weight
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
4 wpg
6 wpg
un
48
72
Time (hpi)


E
IAA ng/g frozen weight
16
14
12
10
8
6
4
2
0
4 wpg
6 wpg
un
48
72
Time (hpi)

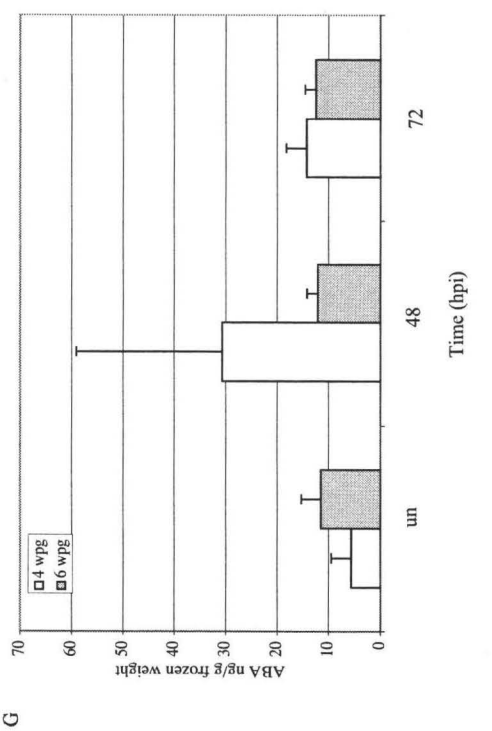
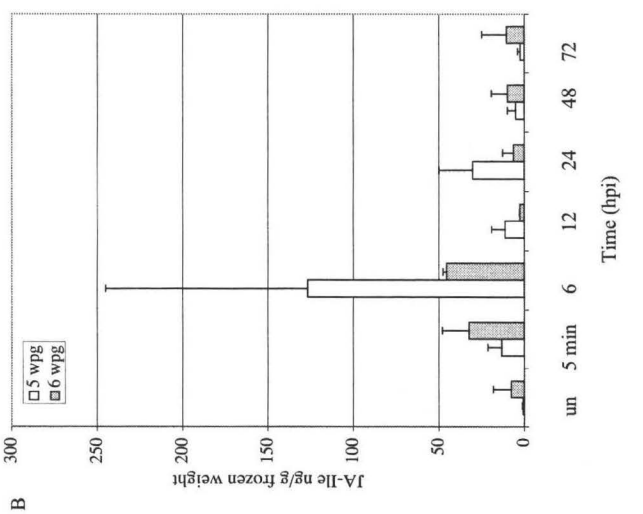
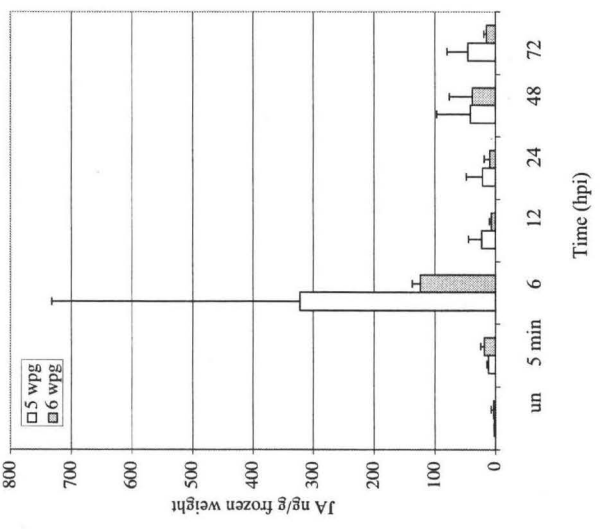
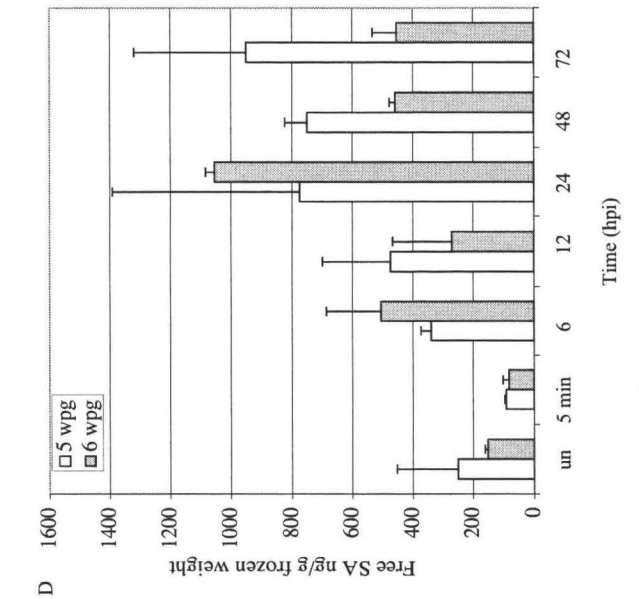
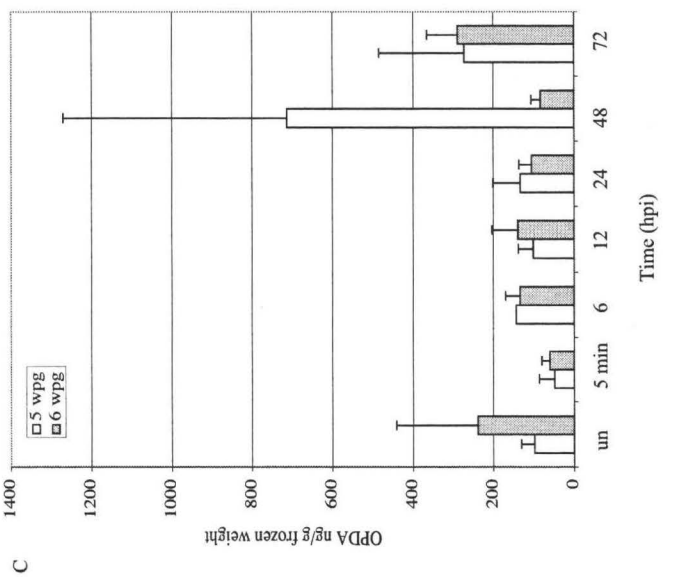

G
ABA ng/g frozen weight
70
60
50
40
30
20
10
0
4 wpg
6 wpg
un
48
72
Time (hpi)

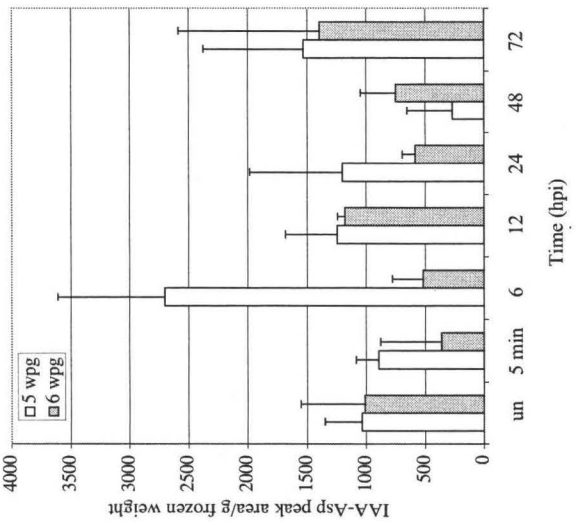
Fig A46: Average (n=2 +/- SD) levels of A) JA, B) JA-Ile, C) OPDA, D) free SA, E) IAA, F) IAA-Asp, and G) ABA in ARR-incompetent plants (5 wpg) and plants exhibiting ARR (6 wpg) plants inoculated with 10^6 cfu ml^{-1} *Pst* in experiments A49 and A47.

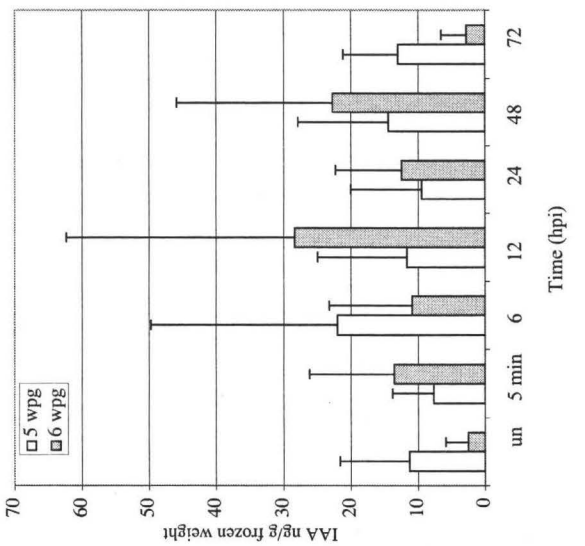

B
JA-Ile ng/g frozen weight
300
250
200
150
100
50
0
□5 wpg
■6 wpg
un
5 min
6
12
24
48
72
Time (hpi)

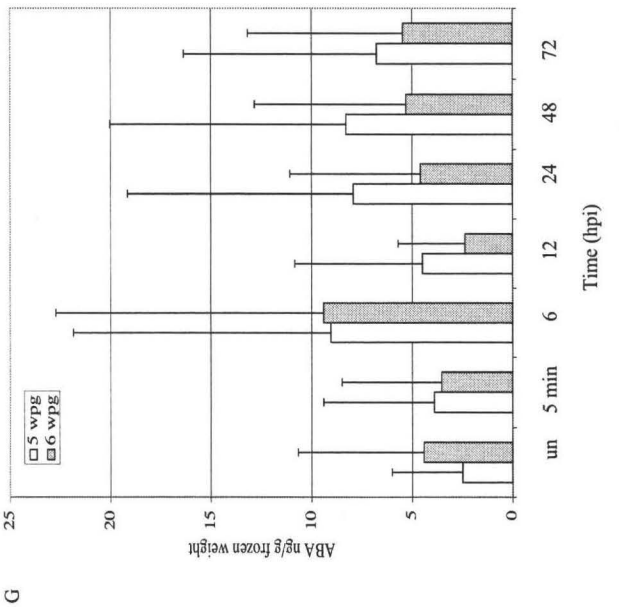

A
JA ng/g frozen weight
800
700
600
500
400
300
200
100
0
□5 wpg
■6 wpg
un
5 min
6
12
24
48
72
Time (hpi)


D
Free SA ng/g frozen weight
1600
1400
1200
1000
800
600
400
200
0
□5 wpg
■6 wpg
un
5 min
6
12
24
48
72
Time (hpi)


C
OPDA ng/g frozen weight
1400
1200
1000
800
600
400
200
0
□5 wpg
■6 wpg
un
5 min
6
12
24
48
72
Time (hpi)

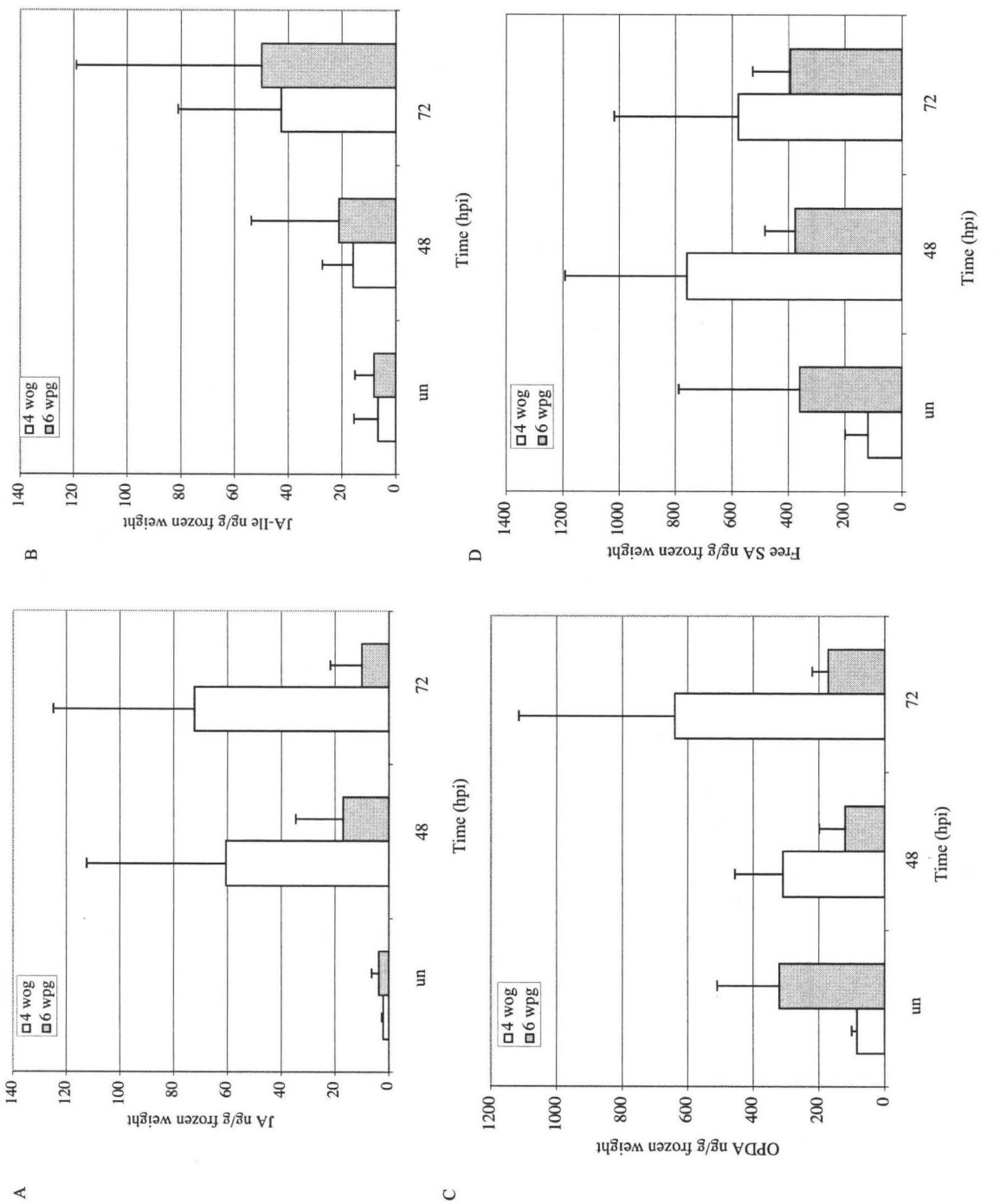

F
IAA-Asp peak area/g frozen weight
4000
3500
3000
2500
2000
1500
1000
500
0
□5 wpg
■6 wpg
un
5 min
6
12
24
48
72
Time (hpi)

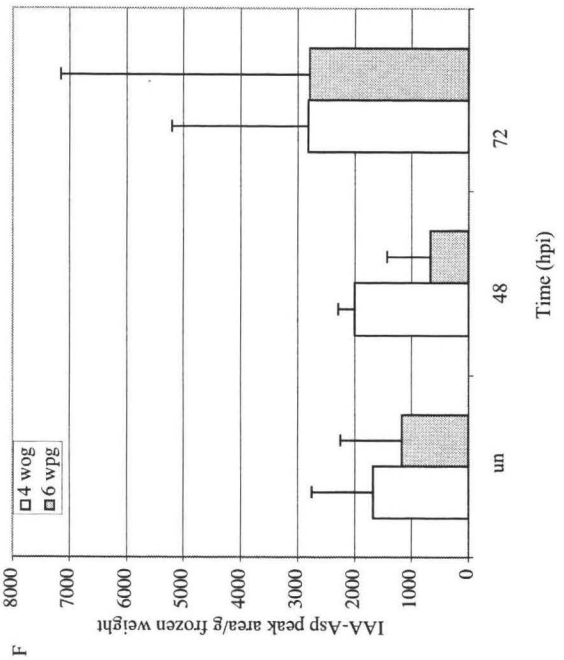

E
IAA ng/g frozen weight
70
60
50
40
30
20
10
0
□5 wpg
■6 wpg
un
5 min
6
12
24
48
72
Time (hpi)

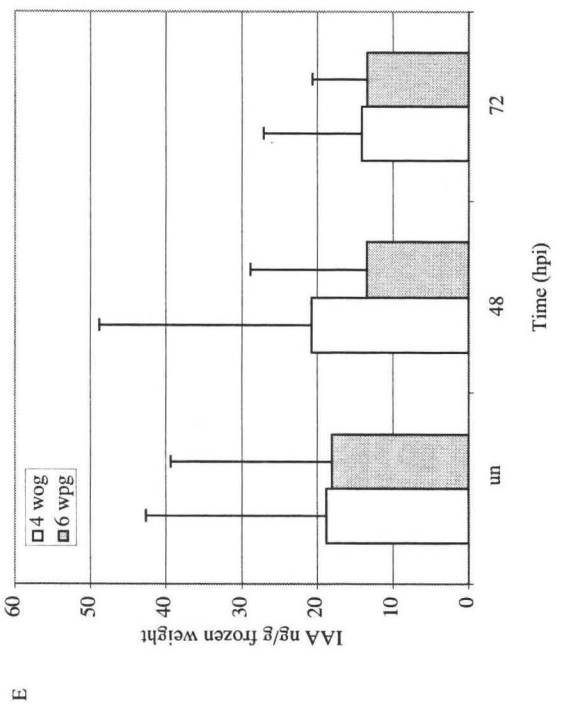

5 wpg
6 wpg
ABA ng/g frozen weight
0
5
10
15
20
25
un
5 min
6
12
24
48
72
Time (hpi)

G

Fig A47: Average (n=2 +/- SD) levels of A) JA, B) JA-Ile, C) OPDA, D) free SA, E) IAA, F) IAA-Asp, and G) ABA in young (4 wpg) and mature (6 wpg) plants inoculated with 10^6 cfu ml^{-1} *Pst* in experiments A43, A49, and A52.


A
JA ng/g frozen weight
4 wog
6 wpg
un
48
72
Time (hpi)
B
JA-Ile ng/g frozen weight
C
OPDA ng/g frozen weight
D
Free SA ng/g frozen weight


F
IAA-Asp peak area/g frozen weight
8000
7000
6000
5000
4000
3000
2000
1000
0
4 wog
6 wpg
un
48
72
Time (hpi)


E
IAA ng/g frozen weight
60
50
40
30
20
10
0
4 wog
6 wpg
un
48
72
Time (hpi)

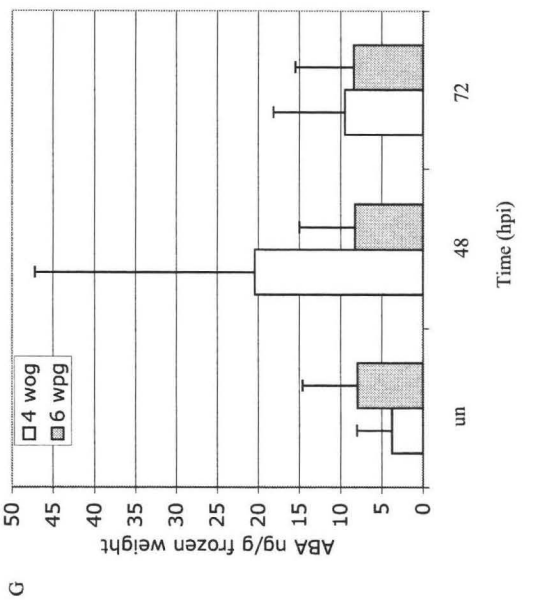
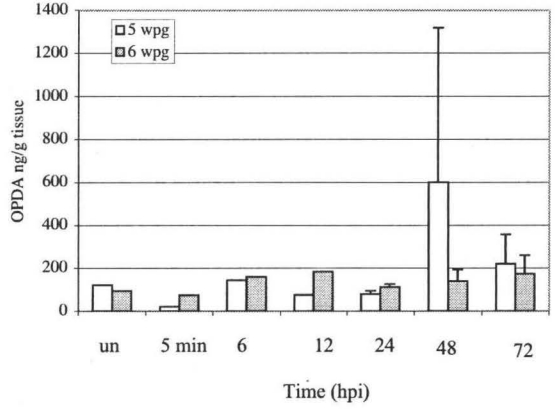
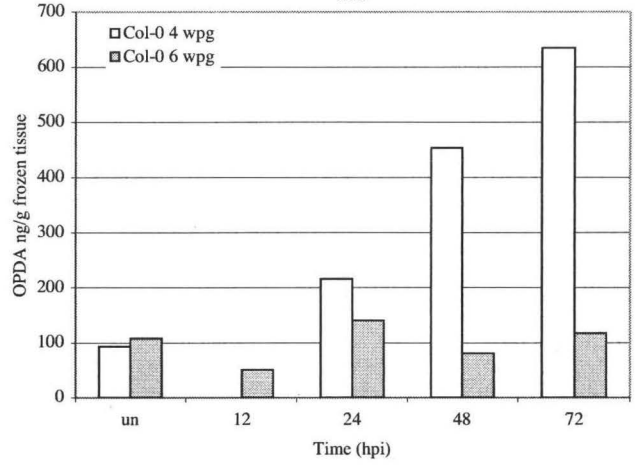

G
ABA ng/g frozen weight
50
45
40
35
30
25
20
15
10
5
0
4 wog
6 wpg
un
48
72
Time (hpi)

Fig A48: OPDA levels in young (4 wpg) and mature (6 wpg) Col-0 inoculated with 10^6 cfu ml^{-1} in experiments A43, A47, A49, and A52. Data points with no standard deviation bars represent one biological replicates. Data points with a standard deviation bar represent the average of two or three biological replicates. Experiment A43 did not include a sample from young plants at 12 hpi.

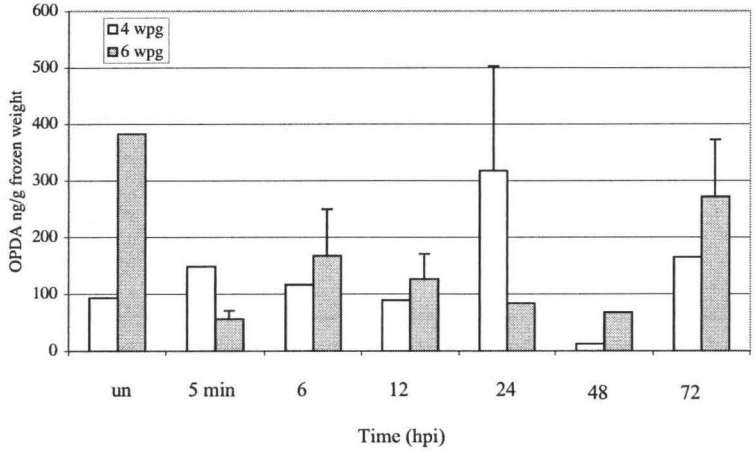
47


5 wpg
6 wpg
OPDA ng/g tissue
1400
1200
1000
800
600
400
200
0
un
5 min
6
12
24
48
72
Time (hpi)

A43


Col-0 4 wpg
Col-0 6 wpg
OPDA ng/g frozen tissue
700
600
500
400
300
200
100
0
un
12
24
48
72
Time (hpi)

49


4 wpg
6 wpg
OPDA ng/g frozen weight
600
500
400
300
200
100
0
un
5 min
6
12
24
48
72
Time (hpi)

52

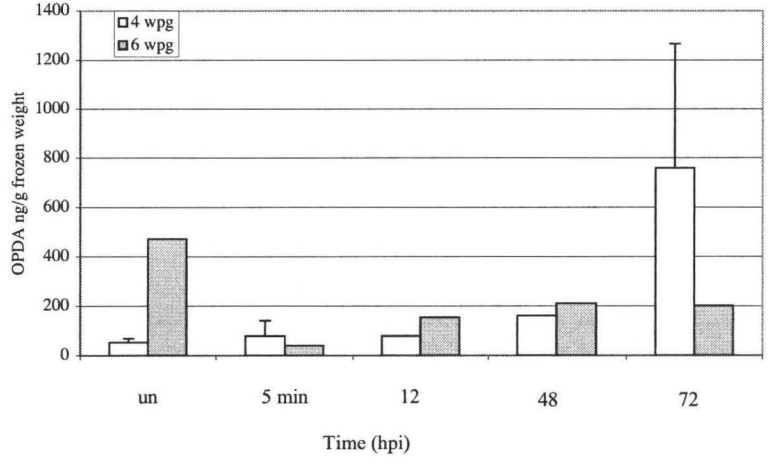
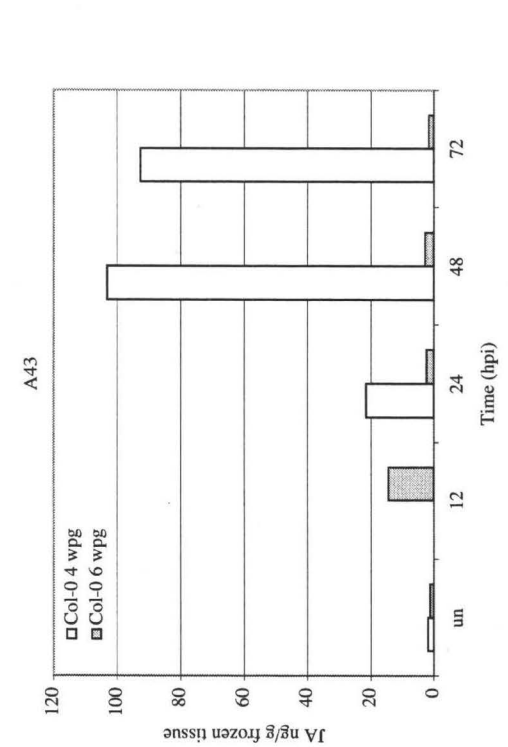
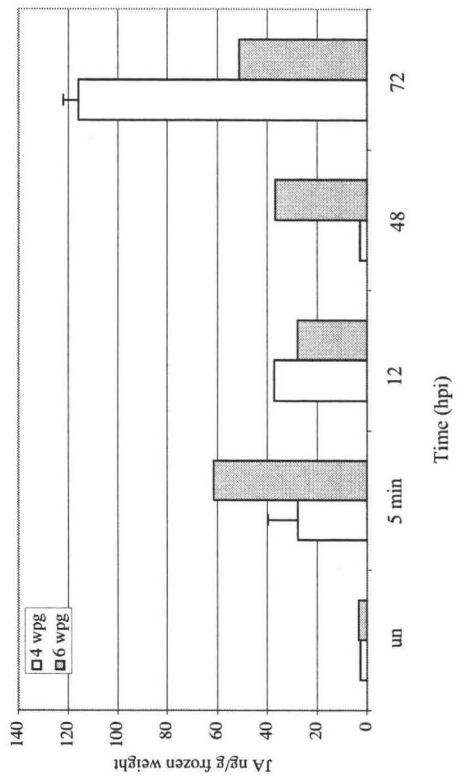
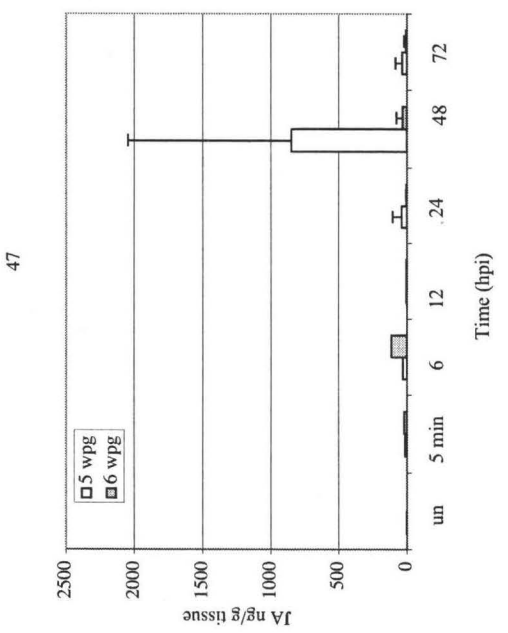

4 wpg
6 wpg
OPDA ng/g frozen weight
1400
1200
1000
800
600
400
200
0
un
5 min
12
48
72
Time (hpi)

Fig A49: JA levels in young (4 wpg) and mature (6 wpg) Col-0 inoculated with 10^6 cfu ml^{-1} in experiments A43, A47, A49, and A52. Data points with no standard deviation bars represent one biological replicates. Data points with a standard deviation bar represent the average of two or three biological replicates. Experiment A43 did not include a sample from young plants at 12 hpi.


A43
Col-0 4 wpg
Col-0 6 wpg
JA ng/g frozen tissue
Time (hpi)
un
12
24
48
72


52
4 wpg
6 wpg
JA ng/g frozen weight
Time (hpi)
un
5 min
12
48
72


47
5 wpg
6 wpg
JA ng/g tissue
Time (hpi)
un
5 min
6
12
24
48
72

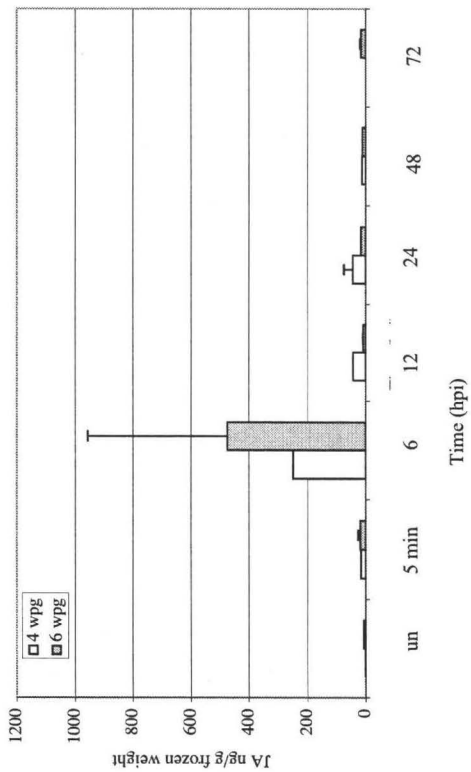

49
4 wpg
6 wpg
JA ng/g frozen weight
Time (hpi)
un
5 min
6
12
24
48
72

Fig A50: SA levels in young (4 wpg) and mature (6 wpg) Col-0 inoculated with 10^6 cfu ml^{-1} in experiments A43, A49, and A52. Data points with no standard deviation bars represent one biological replicates. Data points with a standard deviation bar represent the average of two or three biological replicates. Experiment A43 did not include a sample from young plants at 12 hpi.

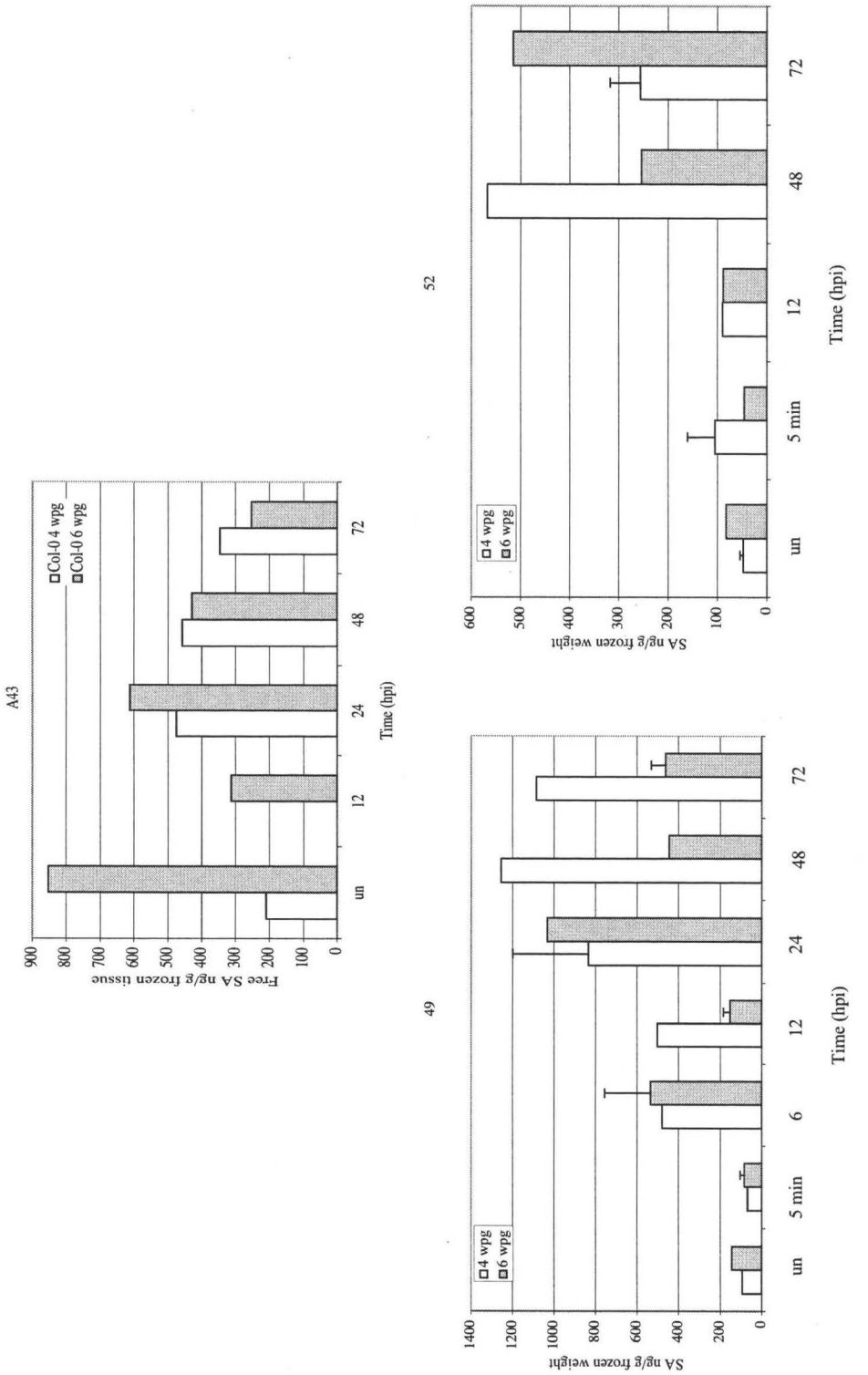
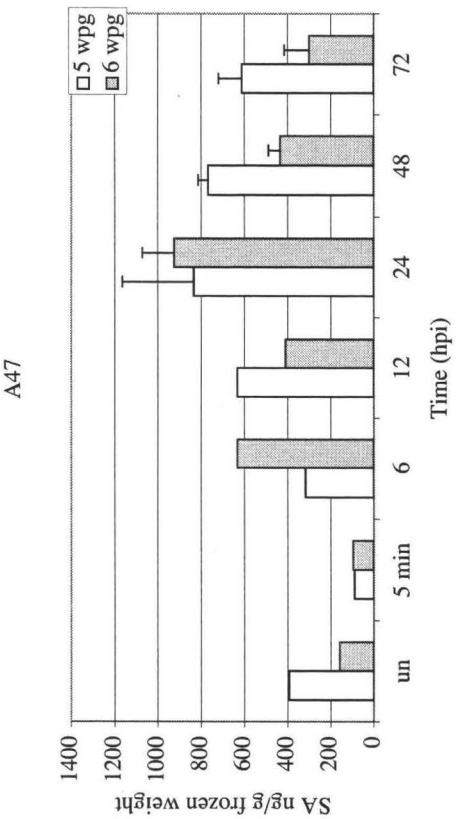
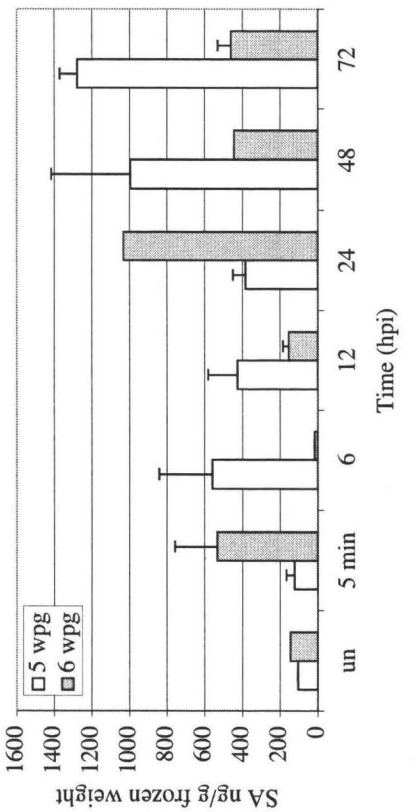

A43
Col-0 4 wpg
Col-0 6 wpg
Free SA ng/g frozen tissue
Time (hpi)
un
12
24
48
72
0
100
200
300
400
500
600
700
800
900
49
4 wpg
6 wpg
SA ng/g frozen weight
un
5 min
6
12
24
48
72
0
200
400
600
800
1000
1200
1400
52
4 wpg
6 wpg
SA ng/g frozen weight
un
5 min
12
48
72
0
100
200
300
400
500
600
Time (hpi)

Fig A51: SA levels in Col-0 ARR-incompetent plants (5 wpg) and Col-0 plants exhibiting ARR (6 wpg) inoculated with 10^6 cfu ml^{-1} in experiments A47 and A49. Data points with no standard deviation bars represent one biological replicates. Data points with a standard deviation bar represent the average of two or three biological replicates.


A47
5 wpg
6 wpg
SA ng/g frozen weight
0
200
400
600
800
1000
1200
1400
un
5 min
6
12
24
48
72
Time (hpi)


A49
5 wpg
6 wpg
SA ng/g frozen weight
0
200
400
600
800
1000
1200
1400
1600
un
5 min
6
12
24
48
72
Time (hpi)

Overexpressing *ANAC055* and *ANAC092* in Arabidopsis

Up-regulation of *ANAC055* and *ANAC092* in the ARR microarray experiment, and the ARR defect exhibited by *anac055* and *anac092* (Chapter 2), suggest that they are positive regulators of ARR. If so, then overexpression of *ANAC055* and *ANAC092* may result in enhanced ARR. To test this idea, transgenic plants expressing *ANAC055* and *ANAC092* under the control of the constitutive 35S Cauliflower Mosaic Virus (35S) promoter could be tested for ARR. To produce these plants the coding sequences of *ANAC055* and *ANAC092* were cloned from cDNA prepared from Arabidopsis leaves into the Gateway pDONR221 plasmid, and the 35S promoter was cloned into the Gateway pDONRP4-P1R plasmid (Invitrogen) (Table A1). All clones were sequenced, confirming that they had the correct insert (see next section).

Table A1. *ANAC055*, *ANAC092*, and the 35S promoter (P) cloned into pDONR vectors

Gene/promoter(P)	Primers[a]	Plasmid[b]	Clone name/expt#[c]
ANAC055 (NAC3)	AttB1NAC3F AttB2NAC3RNS	pDONR221	NAC7-2-4
ANAC092 (NAC5)	AttB1NAC5F AttB2NAC5R	pDONR221	NAC6-3
35SP	AttB435SF AttB135SR	pDONRP4-P1R	NAC14-1, NAC14-3 NAC14-4

[a] Primers used to amplify the target sequence and clone it into the entry plasmid

[b] Invitrogen Gateway plasmid name

[c] This corresponds to the name of the cloning experiment in the lab notebook and the name on the tube that contains the *E. coli* transformed with the respective plasmid stored in the –80 °C freezer

Materials and Methods for Cloning of *ANAC055*, *ANAC092*, and the 35S promoter into Gateway Entry Vectors

RNA was extracted from leaves of young (3 wpg) and mature (6 wpg) Col-0 at 12 and 24 hpi with *Pst* and 10 mM $MgCl_2$ with TRIzol (Invitrogen). DNase treatment was performed with the Ambion DNase Free system (Applied Biosystems), and cDNA was synthesized using the SuperScript II (Invitrogen) reverse transcriptase kit, according to the manufacturers' instructions. The AttBNAC3 and AttBNAC5 primers (The Institute for Molecular Biology and Biotechnology - MOBIX: Table 2.3) were used in a standard PCR reaction with Taq polymerase (Invitrogen) and an annealing temperature of 55 to 60 °C for 35 cycles. The AttB35S primers (Table 2.3) were used to amplify the 35S CaMV promoter from the pMN35Dir1-ssGUS14 vectors present in the Cameron Lab. The PCR mixtures were run on a 1% agarose gel and visualized under ultraviolet light to ensure that only one band was amplified by the primers. The band was cut out and GeneClean II (Qiagen) was used to purify the DNA, according to the manufacturer's instructions. The Gateway Multisite Three Fragment Vector

Construction Kit (Invitrogen) was used to clone the *NAC* PCR product into the pDONR221 entry vector and the 35S promoter PCR product into the pDONRP4-P1R entry vector, according to the manufacturer's instructions. One Shot TOP10 Chemically Competent *Escherichia coli* (Invitrogen) cells were transformed according to the manufacturer's instructions, and plated on lysogeny broth (LB) plates supplemented with kanamycin (10 μg/ml) (Sigma) at 37 °C overnight. The plasmid DNA was extracted from an overnight *E. coli* culture in LB^{kan} media as described in Berghammer et al. (1993), and subsequently cleaned with a standard phenol-chloroform method. Plasmids containing the *ANAC055*, *ANAC092*, and the 35S promoter PCR products were digested with XhoI, Taq1, and Pst1 (Roche), respectively, to ensure that these products were cloned into the vector, then they were sent to The Institute for Molecular Biology and Biotechnology at McMaster University for sequencing.